Immunotherapy of ovarian cancer

Vermeij, Renee

IMMUNOTHERAPY OF OVARIAN CANCER

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RENEE VERMEIJ

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Stellingen behorende bij het proefschrift

Immunotherapy of ovarian cancer

1. Bij patiënten met een recidief van eierstokkanker zijn vaccin geïnduceerde p53-specifieke T-cellen nog steeds meetbaar na behandeling met chemotherapie.
   (Dit proefschrift)
2. Cyclofosfamide gecombineerd met het p53-SLP vaccin resulteert in een toename van het aantal p53-specifieke T-helper cellen in patiënten met eierstokkanker, maar resulteert niet in een daling van het aantal en de activiteit van regulatoire T-cellen.
   (Dit proefschrift)
3. Behandeling van kankerpatiënten met p53-specifieke vaccins veroorzaakt een p53-specifieke afweerreactie, maar heeft geen klinisch effect.
   (Dit proefschrift)
4. Een multi-epitoop vaccin dat p53, SP17, survivin, WT1 en NY-ESO-1 bevat kan als universeel vaccin dienen voor de grote meerderheid van patiënten met eierstokkanker.
   (Dit proefschrift)
5. De werking van een WT1-vaccin voor patiënten met eierstokkanker zal mogelijk verbeteren indien gecombineerd met een behandeling waarbij regulatoire T-cellen worden onderdrukt en aanwezigheid van MHC klasse I wordt gestimuleerd.
   (Dit proefschrift)
6. Expressie van PD-L1 door een tumorcel is niet van prognostische waarde in eierstokkanker.
   (Dit proefschrift)
7. If I had more time, I would have written a shorter letter.
   (M.C. Cicero)
8. De beste manier om chaos te veroorzaken is alles te regelen.
   (K. Boullart)
9. Hoge hakken geven een uitstekend overzicht.
   (M. Alderliesten)
10. Wie niks probeert, die weet niks.
    (J.A.J. Vermeij)

Renee Vermeij
Groningen, 14 december 2011

UMG.

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CONTENTS

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

Ovarian cancer is the most lethal gynaecologic malignancy in the Western World, and represents the fifth leading cause of cancer death among European women [1]. In 2008, 1203 patients were diagnosed and 1021 patients died of ovarian cancer in the Netherlands (Dutch Cancer Registry). Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer as it constitutes 80-90% of cases. In this thesis we will focus on EOC only.

Despite the histological heterogeneity of EOC, most deaths are attributable to the serous type, which comprises 60% of cases and has a propensity to present at an advanced stage [2]. Symptoms of EOC are often non-specific especially at an early stage of disease. Therefore, most EOC patients are asymptomatic until disease has metastasized. Consequently, two-third of all patients is diagnosed with advanced stage disease [1;3]. Although the majority of patients with advanced disease achieve complete clinical response rates due to the current therapy of aggressive cytoreductive surgery and platinum-taxane based chemotherapy, more than 90% develop tumor recurrence. Acquired resistance to further chemotherapy is responsible for treatment failure, resulting in five-year survival rates of only 30% [4]. These records express the need for a new and improved therapy for EOC. The significance of the immune response for the clinical course of EOC has led to attempts to modulate this response with (antigen-specific) immunotherapeutic strategies [5].

$CD8^+$ cytotoxic T-lymphocytes are the most important effector cells for antitumor immune responses. They recognize tumor associated antigen-derived peptides that are processed and presented on the tumor cell surface in association with major histocompatibility complex (MHC) class I molecules, leading to killing of tumor cells [6]. $CD4^+$ T-helper cells recognizing tumor associated antigens presented in association with MHC class II molecules on professional antigen presenting cells, play an important role in orchestrating and sustaining the local immune attack by cytotoxic T-lymphocytes [7;8]. In contrast, $CD4^+$ $FoxP3^+$ regulatory T-cells can also recognize MHC class II molecules thereby impeding antitumor immunity by inhibiting cytotoxic T-lymphocyte activation [9;10]. Memory T-cells can be developed upon antigenic stimulation and can sustain the immune attack to certain antigen presenting (tumor) cells.

In case of EOC, high numbers of intra-epithelial cytotoxic T-lymphocytes are indicative of prolonged survival [11-13]. In contrast, presence of intratumoral regulatory T-cells seem to reduce tumor-specific immunity and results in poorer survival of patients with EOC [14;15]. The latter however is not a general finding for EOC [16;17]. MHC class I down-regulation has been reported to negatively influence survival in EOC [18-21]. Up-regulation of MHC class II on ovarian cancer cells however, results in contrasting findings considering prognosis [20;21].

In EOC a number of tumor-associated antigens have been identified. Tumor-associated antigens in general, are categorized into various classes such as differentiation antigens (tyrosinase and MelanA/MART-1), antigens derived from gene overexpression/amplification (EGFR, Her-2/neu, Survivin, WT1), or mutated self proteins (p53), cancer/testis-antigens (MAGE, SP17, NY-ESO-1), and viral antigens (human papillomavirus and Epstein Barr virus) [22].

In this thesis we focus on active antigen-specific immunotherapy for treatment of EOC patients. Immunotherapy is generally thought of as conferring either passive or active immunity [23]. In passive

immunity antibodies or cytotoxic T-lymphocytes are directly infused, that can exert their activity without recruitment of the host immune system to respond. By active immunity an endogenous immune response is induced, in order to stimulate the immune system to recognize the tumor as non-self [23]. One of the most exciting aspects of stimulating the endogenous immune response is the potential to initiate long-term immunologic memory. Active antigen-specific immunotherapy aims at the induction of cellular immunity in a host that failed to spontaneously develop an effective response [24]. Cellular immunity can be induced to tumor specific well-defined antigens. Several methods have been studied in EOC to stimulate an anti-tumor response, varying from immunization with viral vectors encoding tumor antigens, peptide-pulsed dendritic cells, short peptide vaccines, and vaccines with long peptides spanning a whole protein of interest [5].
Thus far, disappointing clinical results upon immunization with cancer vaccines in EOC patients are observed. An explanation for the lack of efficacy is the presence of immune escape mechanisms counteracting effective T-cell-mediated tumor cell killing. Despite induction of antigen-specific T-helper cells and the recruitment of cytotoxic T-lymphocytes to the tumor, a robust anti-tumor response could not be accomplished due to immune escape mechanisms. T-cells that effectively home to tumor metastases have been shown to be dysfunctional which might be due to immunosuppressive mechanisms in the tumor microenvironment [25]. Several immune escape mechanisms have been identified, such as T-cell anergy due to insufficient B7 co-stimulation, extrinsic suppression by regulatory T-cells, inhibition by ligands such as programmed death-1 ligand 1, metabolic dysregulation by enzymes such as indoleamine-2,3-dioxygenase, and inhibitory factors such as TGF-β, which have all been implicated in the lack of efficacy [26;27]. Immunogenicity of most vaccines needs to be enhanced by improving the robustness of the induced effector T-cell responses and by effectively disrupting the counterproductive immune-regulation [28].
With an improved understanding of mechanisms underlying tumor-induced immune suppression, future therapeutic strategies will likely focus on combined approaches designed to restore antitumor immune responses. Moreover, these combination strategies might help eliminating tumor escape mechanisms and correct tumor-induced immune deviation to enable the host immune system to more effectively control tumor growth.

This thesis aims to provide new clues how to modify vaccination strategies to improve immunogenicity and develop clinical efficacy for treatment of EOC patients.

OUTLINE OF THIS THESIS

Previously, the immunogenicity and clinical efficacy of a p53-specific synthetic long peptide (SLP) vaccine in recurrent ovarian cancer patients has been studied in a phase I/II clinical trial [29]. The p53-SLP vaccine tested consists of ten overlapping synthetic long peptides spanning approximately the whole p53-protein. An advantage using (synthetic) long peptides is that if delivered in the appropriate adjuvant (with antigen presenting cell stimulatory capacity), all potential MHC class I and MHC class II epitopes within the delivered peptides will be processed and presented to host T-cells, thereby enhancing immunogenicity and clinical efficacy. From our previously conducted phase I/II study we know that the p53-SLP vaccine is able to induce p53-specific T-cells. In **chapter 2** it was investigated whether vaccine-induced p53-specific T-cells survive chemotherapy secondary to immunotherapy. Furthermore, long-term clinical benefits after vaccination with synthetic long peptides have been described therefore it was evaluated whether this also applies to ovarian cancer patients treated with the p53-SLP vaccine.

Presence of regulatory T-cells which are known to suppress the immune response may be responsible for the limited clinical effect observed upon immunization with the previously tested therapeutic p53-SLP vaccine in recurrent ovarian cancer patients. Cyclophosphamide in low-dosage is described to selectively deplete regulatory T-cells. In **chapter 3** results of a phase II study on treatment with cyclophosphamide prior to immunization with the p53-SLP vaccine in patients with recurrent ovarian cancer are described. Addition of cyclophosphamide to the p53-SLP vaccine aims to improve immunogenicity and clinical activity by regulatory T-cell depletion. P53-specific immune responses as well as clinical responses were evaluated in patients immunized with the p53-SLP vaccine preceded by cyclophosphamide.

Several clinical trials on p53-vaccines in various cancer types were conducted over the past decade using different vaccination strategies. **Chapter 4** reviews the immunological and clinical responses observed in cancer patients vaccinated with p53 targeting vaccines. Methods and results of the clinical trails were reviewed, categorized by the different vaccination strategies used. Furthermore, several immune-potentiating combination strategies suitable for clinical use are summarized to enhance immunogenicity of the therapeutic p53-vaccines.

Most clinical studies thus far targeted only one tumor-associated antigen, limiting the use of such vaccines to those patients with (over)expression of this specific tumor antigen. As solid tumors often show heterogeneous protein expression, multi-antigen vaccines may have greater therapeutic potential which can compensate for tumor antigen-loss variants. The ability to target multiple antigens may improve the immunogenicity of therapeutic vaccines. Immunization using a cocktail of antigens has been proposed as a "universal" vaccine strategy. **Chapter 5** discusses the use of a cocktail of tumor antigens p53, SP17, survivin, WT1 and NY-ESO-1 for treatment of EOC patients. Presentation of tumor antigens in the context of MHC molecules on tumor cells is critical for the efficacy of targeted immunotherapy. Therefore the expression of tumor antigens p53, SP17, sur-

vivin, WT1 and NY-ESO-1, in the context of MHC class I expression was determined in 270 primary EOC patients.

In **Chapter 6** a promising new tumor-associated antigen Wilms' tumor protein 1 (WT1) in EOC was studied in more detail. Immune response characterization at the primary tumor site generates new clues how to modify therapeutic WT1-based vaccination strategies to improve immunologic and clinical efficacy in EOC. Therefore, WT1 overexpression was related to clinicopathological characteristics, immunological parameters cytotoxic T-lymphocytes, regulatory T-cells, MHC class I, and II molecule expression, and survival in 270 primary EOC patients. Our findings were translated into several immune-potentiating combination strategies suitable for clinical use.

Blockade of immune evading strategies in combination with cancer vaccine treatment might augment immunogenicity and clinical efficacy in cancer patients. Therefore analysis of immune evading strategies at the primary tumor site might be helpful in designing strategies to disrupt this immune inhibition. **Chapter 7** depicts the expression of immune evading programmed death-1 ligands 1 (PD-L1) and 2 (PD-L2) in EOC patients. PD-L1 is described to increase apoptosis of antigen-specific T-lymphocytes contributing to the failure of cytotoxic T-lymphocyte mediated specific immunity. Contrasting results on the prognostic effect of PD-L1/PD-L2 expression in tumor cells are reported. Therefore we performed a survival analysis in a homogeneous cohort of 127 advanced serous EOC patients. Also the influence of PD-L1/L2 expression on tumor-infiltrating T-lymphocytes i.e. cytotoxic-, regulatory- and memory T-lymphocytes and survival was investigated. Blockade of the immune inhibitory PD-1/PD-L1 pathway might be an immune-potentiating strategy for treatment of EOC.

Finally, **chapter 8** discusses and summarizes the results of the research described in this thesis. Suggestions for future studies and considerations for further clinical studies on SLP-vaccines are discussed.

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CHAPTER 2

LONG-TERM CLINICAL AND IMMUNOLOGICAL EFFECTS OF P53-SLP VACCINE IN OVARIAN CANCER PATIENTS

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ABSTRACT

Vaccine-induced p53-specific immune responses were previously reported to be associated with improved response to secondary chemotherapy in small cell lung cancer patients. We investigated longterm clinical and immunological effects of the p53-SLP vaccine in recurrent ovarian cancer patients. Twenty patients were immunized with the p53-SLP vaccine between July 2006 and August 2007. Follow-up information on patients was obtained. Clinical responses to secondary chemotherapy after p53-SLP immunizations was determined by computerized tomography and/or tumour marker levels (CA125). Disease-specific survival was compared with a matched historical control group. Immune responses were analysed by flow cytometry, proliferation assay, IFN-ELISPOT and/or cytokine bead array. Lymphocytes cultered from skin biopsy were analysed by flow cytometry and proliferation assay.

Of twenty patients treated with the p53-SLP vaccine, seventeen were subsequently treated with chemotherapy. Eight of these volunteered another blood sample. No differences in clinical response rates to secondary chemotherapy or disease-specific survival were observed between immunized patients and historical controls ($p=0.925$, resp. $p=0.601$). P53-specific proliferative responses were observed in 5/8 patients and IFN-γ production in 2/7 patients. Lymphocytes cultured from a prior injection site showing inflammation during chemotherapy did not recognise p53-SLP. Thus treatment with the p53-SLP vaccine does not affect responses to secondary chemotherapy or survival, although p53-specific T-cells do survive chemotherapy.

INTRODUCTION

Ovarian cancer, which is generally treated with cytoreductive surgery and platinum-based chemotherapy, is the most frequent cause of death from gynaecological malignancies. In an attempt to improve prognosis by inducing and/or enhancing tumor immune responses, we have recently performed a phase II study with the p53-synthetic long peptide vaccine (p53-SLP) [1]. The vaccine proved safe, well-tolerated and highly immunogenic, but no partial and/or complete clinical responses were observed.

Likewise, many previous p53-based immunotherapeutic strategies have disappointing clinical efficacy, although p53-specific immunity was induced [2–7]. Interestingly, in patients with small cell lung cancer a trend towards an increased response to secondary chemotherapy was observed after immunisation with dendritic cells virally transduced with the wildtype p53 gene [6]. Complete or partial responses to second-line chemotherapy were seen in 75% of p53-responders as opposed to 30% of p53-non-responders. Moreover, this clinical response rate of 75% seen after second-line chemotherapy in patients with immunological responses to the p53-transduced dendritic cells [6] is much higher than observed in historical control groups treated with second-line chemotherapy for progression of disease (6-16%) [8].

Patients with immunological responses to p53-specific immunotherapy may thus be more likely to respond to secondary chemotherapy. Possible explanations for this synergy include 1) up-regulation of p53 in tumour cells in response to chemotherapy thus increasing chances of recognition and destruction by cytotoxic T-cells, and 2) down-regulation of immunosuppressive agents produced by tumour cells, thus enhancing destruction of tumour cells by cytotoxic T-lymphocytes.

We hypothesised that patients treated with the p53-SLP vaccine would have a higher response rate to 'secondary' chemotherapy than generally described for palliative chemotherapy for ovarian cancer. Furthermore, we investigated whether p53-specific immunity previously induced by the p53-SLP vaccine was influenced by 'secondary' chemotherapy.

MATERIAL AND METHODS

ELEGIBILITY CRITERIA

In a phase II study, epithelial ovarian cancer patients were subcutaneoulsy immunized four times with the p53-SLP vaccine [1]. The vaccine consisted of 10 synthetic long overlapping peptides, spanning amino acids 70-248 of the wt-p53 protein. Clinical response to immunizations was determined 6-9 weeks after the last immunization. Subsequent follow-up information for patients who participated in this phase II study was prospectively collected. Patients treated with chemotherapy after immunization were invited to give a blood sample to measure the level of p53-specific immune responses. Written informed consent was obtained specifically for the collection of this additional blood sample.

EVALUATION OF LONG TERM IMMUNOGENICITY

Lymphocytes and sera

Blood for immunological assays was obtained at least 4 weeks after 'secondary' chemotherapy. Serum was isolated from clotted blood and cryopreserved. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Paque density centrifugation and freshly used and/or frozen until use in liquid nitrogen.

Antigens (for immunological assays)

Vaccine peptides were divided into four pools: p1-2 (aa 70-115), p3-4 (aa 102-155), p5-7 (aa 142-203) and p8-10 (aa 190-248). Thirteen overlapping 30-mers spanning the first and last part of wt-p53 protein not included in the vaccine were divided into two pools: aa 1-78 and aa 241-393. Memory recall mix (MRM), a mixture of tetanus toxoid (0.75 limus flocculentius/mL; Netherlands Vaccin Institute, Bilthoven, the Netherlands), tuberculine PPD (0.4 μg/mL; Netherlands Vaccin Institute, Bilthoven, the Netherlands) and *C. albicans* (0.015% Greenlabs, Lenoir) was used as a positive control.

IFN-γ ELISPOT assay

Cryopreserved PBMC obtained before immunization, after immunization and after subsequent chemotherapy were available for 7 patients. P53-specific responses in cryopreserved PBMC obtained before immunization, after four immunizations and after chemotherapy of a single patient were simultaneously determined by IFN-γ ELISPOT as previously described [5,9]. PBMC were stimulated with vaccine and non-vaccine p53-peptide pools (10 μg/peptide/mL) or MRM (1:50). A response was considered p53-specific if [(mean number of spots in experimental wells) – (mean number of spots in medium + 2 x SD)] ≥ 10 spots / 10^5 PBMC. A vaccine-induced response was defined as a p53-specific response which exceeded the pre-existing immune response at least three-fold [10].

Flow cytometry

PBMC were evaluated for CD3, CD4, CD8, CD19, CD56 (IQ Products, Groningen, the Netherlands) and FoxP3 expression (eBioscience, San Diego, CA) by flow cytometry (FACSCalibur from BD Biosciences, Erembodegem, Belgium) according to manufacturer's instructions. Skin biopsy derived lymphocytes were analysed for CD4, CD8, and Foxp3 expression.

Proliferation assays

Freshly isolated PBMC were stimulated with vaccine and non-vaccine p53-peptide pools (10 μg/peptide/mL) or MRM (1:156) as previously described [10]. Proliferation was considered p53-specific if the stimulation index ≥ 3 and [(mean cpm in experimental well) – (mean cpm in medium + 3x SD medium)] ≥ 0. A vaccine-induced response was defined as a p53-specific response with [(mean cpm in medium + 3x SD medium after treatment) / ((mean cpm in medium + 3x SD medium before immunization)] ≥ 2.

Cytokine bead array

Production of Th1 cytokines IL-2, IFN-γ and TNF-α, Th2 cytokines IL-4, IL-5 and IL-10 was evaluated for three patients. Supernatants of proliferation assays were used in a cytokine bead array (LINCO-*plex kit*, Linco Research, St. Charles, MO) as according to a previously validated standard operating procedure [9]. P53-specific cytokine production was defined as concentration of cytokine ≥ 2 medium control and if concentration ≥ 100 pg/mL (IFN-γ), or ≥ 10 pg/mL (other cytokines).

EVALUATION OF LONG-TERM CLINICAL ACTIVITY

Historical control group

To evaluate the effect of p53-SLP treatment on subsequent chemotherapy and survival, a historical control goup was formed with three control patients for each patient treated with the p53-SLP vaccine. Historical controls were obtained from an anonymous pass-word protected database containing clinicopathological and follow-up data of all epithelial ovarian cancer patients treated with primary debulking surgery according to standard treatment protocols by gynecological oncologists of the University Medical Center Groningen (Groningen, The Netherlands) between May 1985 and May 2006. As no patient identity can be eluded from this computerized database, no further approval from our Institutional Review Board was required for the use of these historical control patients according to Dutch law. P53-SLP treated patients were matched with historical controls based on FIGO stage, histological tumour type, amount of residual disease after primary debulking surgery, histological grade (in order of importance).

EVALUATION OF CLINICAL RESPONSES TO CHEMOTHERAPY

For historical control patients, tumour response to secondline chemotherapy was evaluated based on CA-125 levels and reports of imaging when available. For p53-SLP treated patients, tumour

response to post-immunotherapy chemotherapy was evaluated by serum CA-125 levels (GCIG criteria [11]) and computerized tomography assessed according to RECIST criteria [12] by an experience radiologist (RW)).

STATISTICAL ANALYSES

Differences in PBMC composition of cryopreserved samples and cytokine levels were evaluated with the Wilcoxon signed rank test. Whether matching resulted in similar distributions of clinicopathological characteristics was evaluated by likelihood ratio statistics (categorical variables) or independent samples t-test (normally distributed continous variables). Differences between historical controls and p53-SLP vaccine treated patients in response rates to chemotherapy for first recurrence were evaluated with Chi-square test. Survival differences were plotted using Kaplan-Meier curves and tested by Log-Rank test. Disease-specific survival was defined as time of diagnosis to date of death due to ovarian cancer, treatment related fatalities or last-follow-up.
All analyses were performed using SPSS version 16.0.2 software package for windows (SPSS Inc., Chicago, USA). P values <0.05 were considered significant (tested 2-sided).

RESULTS

PATIENTS

Twenty patients were treated with the p53-SLP vaccine [1]. Three patients did not receive subsequent chemotherapy (P01, P12, P14). Responses to p53-SLP treatment and post-immunotherapy chemotherapy are shown in table 1. Eight patients consented to the donation of an additional blood sample after chemotherapy. No differences in clinicopathological characteristics of immunized patients and matched historical controls were observed (table 2).

Table 1 Overview of administered p53-SLP therapy and secondary chemotherapy as clinical responses to therapy

Patient	Number of p53-SLP immunizations	Response to p53-SLP	Type of post-immunotherapy chemotherapy	Response to post-immuno-therapy chemo-therapy#	Post-chemo-therapy PBMC sample
P01	4	PD	-	n.a.	n.a.
P02	4	PD	C	PR	No
P03	4	PD	LP	SD	No
P04	2	PD	C, P	PR	No
P05	4	PD	C	PR	No
P06	4	PD	C, P	PR	Yes
P08	4	PD	LP	PD	No
P09	4	PD	C, P	PR	Yes
P11	4	PD	C, D	PD	Yes
P12	2	PD	-	n.a.	n.a.
P13	4	PD	LP	PR	No
P14	4	PD	-	n.a.	n.a.
P15	4	PD	C, P	PR	No
P17	4	SD	C, D	CR	Yes
P18	4	PD	C	PD	No
P19	4	PD	C, D	PR	Yes*
P20	4	PD	C, LP	PR	Yes
P21	4	PD	C, P	PR	No
P22	4	PD	C, D	PR	Yes*
P23	4	SD	LP	PD	Yes

C = carboplatin, D = docetaxel, P = paclitaxel, LP = liposomal doxorubicin, CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, n.a. = not applicable; #based on evaluation of CA125 levels and CT-scans, except for P04/P05 only CA125 and P23 only CT-scan; *second additional sample obtained at time of reactivation of prior injection sites during tertiary chemotherapy for a second recurrence.

Table 2 Clinicopathological characteristics of p53-SLP treated patients and matched historical controls

	P53-SLP (n=20)		Historical controls (n=60)		p-value
Age at diagnosis					
Mean (SD)	52.4	(8.8)	58.7	(11.8)	0.032
FIGO stage					
IC	1	(5.0%)	3	(5.0%)	0.219
IIc	1	(5.0%)	3	(5.0%)	
IIIB	2	(10.0%)	0	(0.0%)	
IIIC	14	(70.0%)	48	(80.0%)	
IV	2	(10.0%)	6	(10.0%)	
Histology					
Serous	13	(65.0%)	39	(65.0%)	1.000
Mucinous	1	(5.0%)	3	(5.0%)	
Endometrioid	3	(15.0%)	9	(15.0%)	
Clear cell	3	(15.0%)	9	(15.0%)	
Differentiation					
Grade I	2	(10.0%)	6	(10.0%)	0.263
Grade II	5	(25.0%)	20	(33.3%)	
Grade III	13	(65.0%)	29	(48.3%)	
unknown	0	(0.0%)	5	(8.3%)	
Residual disease					
<2 cm	10	(50.0%)	29	(48.3%)	0.808
≥2 cm	6	(30.0%)	22	(36.7%)	
unknown	4	(20.0%)	9	(15.0%)	

FIGO = international federation of Gynecology and Obstetrics

Persistent p53-specific proliferative T-cell responses after chemotherapy

P53-specific proliferative responses were observed in 5/8 patients (63%) after post-immunotherapy chemotherapy as measured by proliferation assay (figure 1). In three patients (P17, P20, P23) responses present after the fourth immunization were no longer detectable after subsequent chemotherapy, whereas the opposite held for P09. Persisting responses were frequently more pronounced after chemotherapy than after the fourth immunization. Interestingly, after chemotherapy

proliferative responses to aa 241-393, part of the p53-protein not covered by the p53-SLP vaccine, increased in number as well as strength.

An IFN-γ ELISPOT was performed for seven patients to evaluate whether these p53-specific cells were IFN-γ producing Th1 type cells (figure 2). Comparable to the previously published results [1], no differences existed in composition of cells obtained before immunization, after immunization and after chemotherapy as analysed simultaneously by flow cytometry (data not shown). After chemotherapy, two patients (29%) showed p53-specific IFN-γ production in response to stimulation of PBMC with vaccine peptides p3-p4 and p8-p10 (P06, P20), whereas no such responses were observed in these patients after four immunizations. In patients with responses against vaccine peptides after immunotherapy (P11, P17, P22) no IFN-γ producing T-cells could be detected after subsequent chemotherapy. IFN-γ production by cells that proliferate in response to stimulation with p53-specific peptides therefore does not seem to be augmented by chemotherapy after immunotherapy with the p53-SLP vaccine.

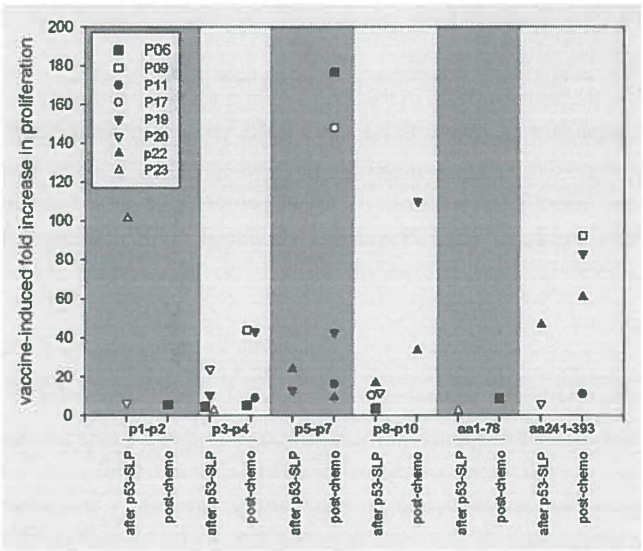

Figure 1 Vaccine-induced p53-specific immune responses in PBMC of ovarian cancer patients immunized with the p53-SLP vaccine as analysed by proliferation assay after four immunizations and after subsequent chemotherapy. PBMC were stimulated with vaccine peptides p1-p2, p3-p4, p5-p7, p8-p10 or with overlapping 30-mers spanning the first and the last part of the wt-p53 protein not included in the vaccine. All measurements were performed in octuplicate. Responses are depicted as the fold increase in mean of corrected p53-induced proliferation after four immunizations or chemotherapy compared to corrected pre-immunization p53-specific proliferation. Only p53-specific responses are shown. For P11 no sample was available for testing after four immunizations.

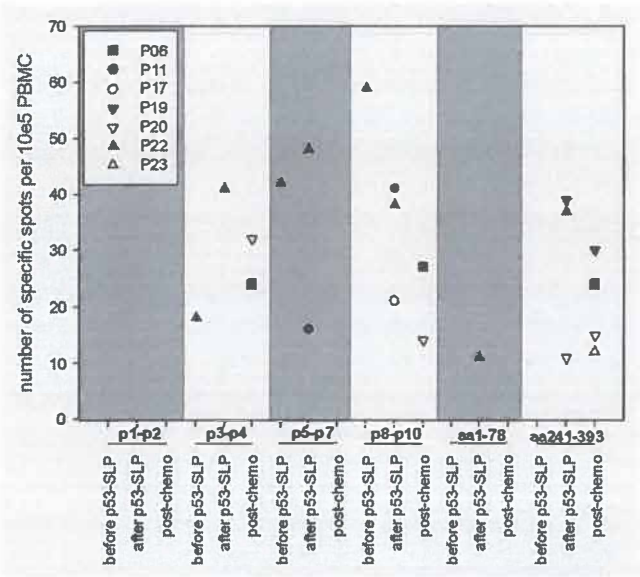

Figure 2 Vaccine-induced p53-specific immune responses in PBMC of ovarian cancer patients immunized with the p53-SLP vaccine as analysed by IFN-γ ELISPOT. PBMC were stimulated with vaccine peptides p1-p2, p3-p4, p5-p7, p8-p10 or with overlapping 30-mers spanning the first and the last part of the wt-p53 protein not included in the vaccine. All measurements were performed in quadruple. Only p53-specific responses are depicted. P09 was not evaluated due to insufficient PBMC.

Th1/Th2 cytokine production is not altered by post-immunization chemotherapy

Th1 and Th2 cytokine production was evaluated in three patients. P53-specific cytokine production was observed at all timepoints. Although overall vaccine-induced cytokine concentrations do not seem to be different between post-immunotherapy and post-chemotherapy samples in this small sample group of patients, p53-specific production of Th2 cytokines (i.e. IL5, IL-10) as well as Th1 cytokine TNF-α seemed to be more common after post-immunotherapy chemotherapy (figure 3).

REACTIVATION OF INJECTION SITES IS NOT CAUSED BY P53-SPECIFIC T-LYMPHOCYTES

Reactivation of prior injection sites during chemotherapy was reported by several patients (figure 4). For two patients (P19, P22) an additional blood sample was obtained for IFN-γ ELISPOT at the time of reactivation during chemotherapy for a second recurrence, in addition to the sample obtained after the first course of post-immunotherapy chemotherapy. Similar to the results after the first course of post-immunotherapy, no p53-specific responses were observed for P19 at the time of reactivation of the vaccine injection sites. Although P22 had p53-specific responses against p3-

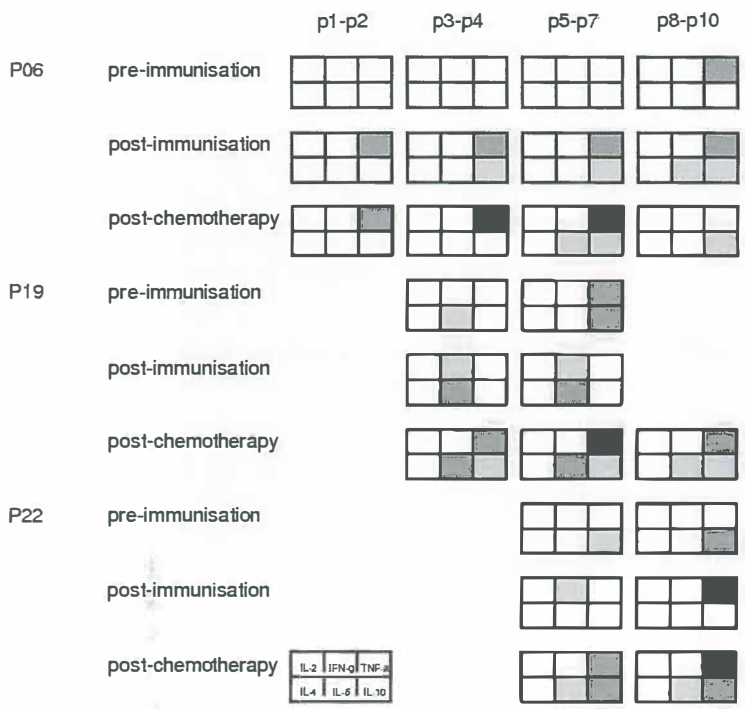

Figure 3 Cytokine production by p53-specific T-cells prior to immunization, after four immunizations and after subsequent chemotherapy. Cytokines were measured in supernatants of cultures with a p53-specific response as measured by proliferation assay. P53-specific responses in proliferation assays are represented by a box. P53-specific production of a cytokine is indicated by a filling (yellow: 1-5x cut-off; orange: 5-15x cut-off; red: 15-50x cut-off; and dark red: >50x higher than cut-off). Median and ranges of vaccine-induced cytokine production after four immunizations and after chemotherapy for Th1 cytokines were IFN-γ 158 [123-486] pg/ml, resp. no vaccine-induced production after chemotherapy; TNF-α 163 [79-1255] resp. 120 [59-305] pg/ml; and for Th2 cytokines IL-5 37 [12-114] resp. 35 [21-97] pg/ml; IL-10 78 [17-146] resp. 36 [18-89] pg/ml.

p4, p5-p7 and p8-p10, only responses against p8-p10 could be considered vaccine-induced (43, 57 and 189 specific spots / 10^5 cells respectively).

P22 also consented to a skin biopsy taken from a reactivated immunization site. This biopsy was used for the culture of T-lymphocytes as previously described [1], yielding 12.3 x 10^6 lymphoyctes after 4 weeks of culturing ($CD4^+$ 15%, $CD8^+$ 5%). Cells were subsequently used for a 6-day proliferation assay. The lymphocytes cultured from the biopsy taken from the reactivated injection site were not p53-specific, indicating that reactivation of prior injection sites is not a p53-specific event (data not shown).

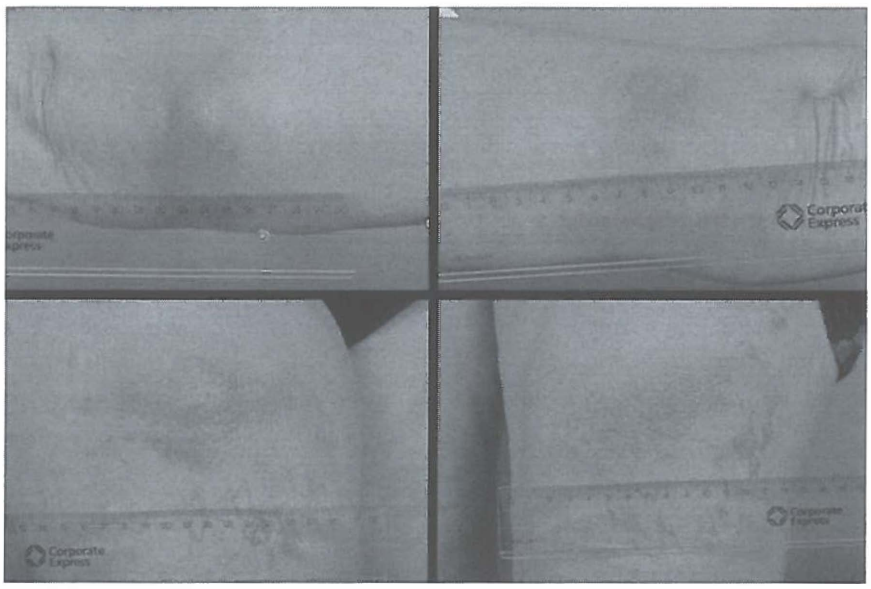

Figure 4 Spontaneous loco-regional inflammatory responses during chemotherapy for secondary recurrence at all four injection sites.

LONGTERM CLINICAL ACTIVITY

Information on clinical response to secondline chemotherapy was available for 30 historical controls. Response rates to secondline chemotherapy were similar for p53-SLP vaccine treated patients and historical controls (CR/PR 60.0% vs. 61.5%, p=0.925). Likewise, median disease-specific survival did not differ between p53-SLP treated ovarian cancer patients and historical controls (median 44.0 vs. 47.4 months, p=0.601). Inclusion of only those patients who received all four p53-SLP SLP immunizations or exclusion of patients not treated with chemotherapy after immunotherapy did not result in differences in survival either.

DISCUSSION

The p53-SLP vaccine was recently shown to induce p53-specific T-cell responses in ovarian cancer patients [1]. We investigated longterm immunological and clinical effects of the p53-SLP vaccine as it has been suggested that responses to chemotherapy might improve in patients with vaccine-induced immune responses [6]. Despite the presence of p53-specific immune responses in patients treated with the p53-SLP vaccine, neither clinical responses to chemotherapy for recurrent disease nor survival differed from the response rates and survival of historical controls. After chemotherapy for recurrent disease subsequent to p53-SLP immunotherapy, p53-specific immune responses could be detected in 6/8 patients willing and able to give an additional blood sample. Moreover, after chemotherapy epitope spreading was observed as proliferative responses to parts of the p53-protein not covered by the p53-SLP vaccine increased in number as well as strength. Although reactivation of inflammatory reactions at prior injection sites during chemotherapy for a second recurrence was accompanied by circulating p53-specific T-cells recognizing p8-p10 in one patient, no locally active p53-specific T-cells could be detected.

A trial of p53 immunization in small cell lung cancer patients showed a trend towards improved response rates to secondary chemotherapy for immunological responders [6]. As there was no distinct group of non-responders in our previous trial [1], a similar comparison between immunologial responders and non-responders could not be made. We therefore compared clinical response rates to chemotherapy for recurrent disease with a historical control group. No differences in response rates to chemotherapy were observed, which may be attributed to several causes. Firstly, although an attempt was made to match p53-SLP treated patient with historical controls based on some well-known prognostic factors, such retrospective comparisons remain prone to selection bias. To obtain a truly reliable insight in the effect of p53-SLP treatment on chemotherapy and survival, a randomized controlled trial should be performed with patients allocated to p53-SLP treatment or a control arm receiving no or placebo treatment. Secondly, one could argue that the likelihood of improved clinical responses after p53-SLP treatment is limited as immunization with the p53-SLP vaccine was shown to induce predominantly Th2 $CD4^+$ T-cells, which are less likely to contribute to effective anti-tumour responses than Th1 $CD4^+$ T-cells [1]. Moreover, after chemotherapy, the number of patients with IFN- producing p53-specific T-cells was lower than the number of patients with p53-specific proliferating T-cells. Although the latter results do not reach statistical significance in this small study, it is noteworthy that proliferative responses were frequently more pronounced after chemotherapy. Within the limitations of our study, the data suggest that especially IFN-producing Th1 $CD4^+$ T-cells are prone to destruction by chemotherapy, whereas Th2 $CD4^+$ T-cells seem to be less affected. Such an effect of chemotherapy on Th1 and Th2 T-cells was previously described for breast cancer patients [13]. Corroborating cytokine profiles were observed in the current study, with increased frequency of Th2 cytokine production after chemotherapy.

A separate, although related observation was the rise in circulating p53-specific T-cells after chemotherapy, which might next to boosting of proliferative responses by chemotherapy be attributable to the loss of activation markers necesarry for extravasation and recruitment of lymphocytes to affected tissues. This would result in increased numbers of circulating p53-specific lymphocytes,

which corresponds to observations in cervical cancer patients in whom responses increased in time withouth additional immunizations or cytotoxic treatment [10].

Interestingly, several patients reported reactivation of immunization sites during chemotherapy for recurrent disease. P53-specific immune responses in blood samples obtained were observed in one of two patients. In a skin biopsy of the injection site from this patient, no p53-specific T-cells could be detected. This suggests that the inflammatory reactions at the immunization sites during chemotherapy may be an immune responses enhanced by Montanide ISA51, the adjuvant used in the p53-SLP vaccine. Next to its depot function, this water-in-oil emulsion allows slow release of antigens, and has been reported to promote inflammation (innate immune responses) and recruitment of antigen presenting cells as well as lymphocytes (adaptive immune responses) [14].
In summary we show that vaccine-induced p53-specific T-cells can still be detected after chemotherapy, but our results indicate that immunotherapy of recurrent ovarian cancer with the p53-SLP vaccine does not affect responses to subsequent chemotherapy or prognosis.

ACKNOWLEDGMENTS

We thank all patients who were willing to donate additional blood samples and/or skin biopsies. This project was funded in part by the Dutch Cancer Society (Amsterdam, The Netherlands; grant 2007-3919) and ISA Pharmaceuticals B.V. (Bilthoven, The Netherlands). C. Melief is a part-time employee of ISA Pharmaceuticals B.V..

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CHAPTER 3

POTENTIATION OF A P53-SLP VACCINE BY CYCLOPHOSPHAMIDE IN OVARIAN CANCER, A SINGLE ARM PHASE II STUDY

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ABSTRACT

The purpose of the current phase II single-arm clinical trial was to evaluate whether pre-treatment with low-dose cyclophosphamide improves immunogenicity of a p53-synthetic long peptide (SLP) vaccine in patients with recurrent ovarian cancer. Ovarian cancer patients with elevated serum levels of CA-125 after primary treatment were immunized four times with the p53-SLP vaccine. Each immunization was preceded by administration of 300mg/m^2 intravenous (i.v.) cyclophosphamide, as a means to affect regulatory T-cells (Tregs). Vaccine-induced p53-specific IFN-γ producing T-cells evaluated by IFN-γ ELISPOT were observed in 90% (9/10) and 87.5% (7/8) of evaluable patients after two and four immunizations, respectively. Proliferative p53-specific T-cells, observed in 80.0% (8/10) and 62.5% (5/8) of patients, produced both T-helper 1 (Th1) and T-helper-2 (Th2) cytokines. Cyclophosphamide induced neither a quantitative reduction of Tregs determined by $CD4^+FoxP3^+$ T-cell levels, nor a demonstrable qualitative difference in Treg function, tested in vitro. Nonetheless, the number of vaccine-induced p53-specific IFN-γ producing T-cells was higher in the current study, compared to a study in which a similar patient group was treated with p53-SLP monotherapy ($p \leq 0.012$). Furthermore, the strong reduction in the number of circulating p53-specific T-cells observed previously after four immunizations was currently absent. Stable disease was observed in 20.0% (2/10) of patients and the remainder of patients (80.0%) showed clinical, biochemical and/or radiographic evidence of progressive disease.

The outcome of this phase II trial warrants new studies on the use of low-dose cyclophosphamide to potentiate the immunogenicity of the p53-SLP vaccine, or other anti-tumor vaccines.

INTRODUCTION

Epithelial ovarian cancer is the leading cause of death from gynecological malignancies in Western countries. The observation of improved prognosis in patients with intra-tumoral T-lymphocytes [1-3] has encouraged the development of immunotherapy for ovarian cancer. Immunotherapy aims to enhance anti-tumor immunity to eliminate malignant cells.

A potential target for cancer immunotherapy is the tumor-suppressor protein p53. Mutation of the p53 gene is a frequent event in human oncogenesis [4;5] which leads to persistent overexpression of p53 in 50-60% of ovarian cancers [6;7]. Therefore, the majority of ovarian cancer patients might benefit from p53 directed immunotherapy. Despite the fact that p53 is a self-protein, studies both in mice and patients demonstrated that p53-specific immune responses can be induced [8-11]. Therefore, we and others recently reported on clinical trials with vaccines targeting p53 [12;13].

Although most immunotherapeutic strategies for ovarian cancer treatment investigated so far are capable of inducing antigen-specific immunity, unequivocal clinical benefit for these patients has not yet been demonstrated [14]. Similarly, we showed that a p53-synthetic long peptide (SLP) vaccine induces p53-specific T-cell responses in ovarian cancer as well as colorectal cancer patients, but observed no clinical benefit [12;15;16]. The observed lack of clinical efficacy may be partly attributed to the presence of regulatory T-cells (Tregs). In ovarian cancer, the presence of Tregs and especially the ratio between Tregs and effector T-cells have been shown to be important for prognosis [2;3;17].

Given the observed effects of cancer immunotherapy on immune inhibitory Tregs [18], strategies to eliminate or suppress Tregs are being explored in an attempt to improve clinical efficacy of cancer immunotherapy. One of these strategies is treatment with low-dose cyclophosphamide, a well-known cytotoxic agent that was widely applied in the treatment of ovarian cancer until the introduction of platinum-based chemotherapy [19]. Dosages used in combination with immunotherapy are generally insufficient for cytotoxic reductions of tumor burden, but reduce Treg numbers and impair their function without deleting other immune cells [20-22]. In a murine model, we showed a synergistic effect of cyclophosphamide and wildtype p53-specific cytotoxic $CD8^+$ T-lymphocytes in growth control of p53-overexpressing tumors [23]. Its putative Treg-depleting and immune-potentiating characteristics make cyclophosphamide an interesting candidate drug for the elimination of Treg when combined with the p53-SLP vaccine, tested in our previous phase I/II study in ovarian cancer patients [12].

We report the results of a phase II single-arm study combining the p53-SLP vaccine with cyclophosphamide in an attempt to improve immunogenicity and deplete the number of Tregs determined by $CD4^+FoxP3^+$ T-cell levels. Ten ovarian cancer patients with (biochemical evidence) of recurrence of disease were immunized four times with the p53-SLP vaccine. Each immunization was preceded by the administration of low-dose cyclophosphamide. Next to immunological responses, clinical activity and safety were monitored.

MATERIALS AND METHODS

The study protocol for this uncontrolled phase II study was approved by the Central Committee on Research Involving Human Subjects (CCMO; NL21308.000.07) and conducted in compliance with the Declaration of Helsinki. All patients gave written informed consent. The trial was registered at the US National Institutes of Health clinicaltrials.gov (NCT00844506) and in the Netherlands National Trial Register (NTR1407). An independent agency (Trial Coordination Center, Groningen, The Netherlands) was contracted to monitor adherence to GCP principles.

PARTICIPANTS

Epithelial ovarian cancer patients with (biochemical) evidence of recurrent disease after prior cytoreductive surgery and chemotherapy, who were not eligible for renewed chemotherapy, were included. Other inclusion criteria were an adequate hepatic and renal function. Additional in- and exclusion criteria were similar to our previous study [12]. Primary tumors were evaluated for p53 expression (BP53-12-1, 1:800, Biogenex) for study cohort characterization purpose only, as patients could participate in the study despite their p53-expression status. Tumors with > 50% moderate or strong immunostaining were considered to have p53-overexpression [7;24].

VACCINE AND TREATMENT SCHEME

In this phase II single-arm clinical trial recurrent ovarian cancer patients were treated with the p53-SLP vaccine combined with low-dose cyclophosphamide. The p53-SLP vaccine consists of 10 synthetic 25-30 amino acid long overlapping peptides (spanning amino acids 70-248 of the wt-p53 protein) dissolved in dimethyl sulfoxide (final concentration 20%) admixed with 20 mM phosphate buffer (pH 7.5) and emulsified with an equal volume of Montanide ISA-51 [12]. The p53-SLP vaccine was administered at a dose of 300 μg/peptide, subcutaneously four times with a three-week interval. Two days prior to each immunization, patients were given an intravenous cyclophosphamide infusion (300mg/m^2 in 30 minutes). Immunizations preceded by cyclophosphamide were administered between October 2008 and July 2009.

STUDY OBJECTIVES

The current study was primarily designed to evaluate whether pre-treatment with cyclophosphamide would (1) improve immunogenicity of the p53-SLP vaccine, and (2) affect regulatory T-cells, determined by CD4$^+$FoxP3$^+$ T-cell levels. Secondary objectives were (1) evaluation of the clinical activity induced by the p53-SLP vaccine preceded by cyclophosphamide infusion, and (2) evaluation of the safety of the p53-SLP vaccine when preceded by cyclophosphamide infusion. Due to a lack in the decrease of FoxP3$^+$ T-cell levels measured, being one of the primary endpoints of the interim analysis of 10 patients, the study stopped. Primary endpoint data of the current single-arm study were compared to data obtained in our previously conducted phase I/II clinical trial on the p53-SLP vaccine [12]. Standard operating procedures (SOPs) used for study material analysis were the same in both trials.

OUTCOME MEASURES

(1) IMMUNOMONITORING

Lymphocytes and sera

Blood for immunological assays was obtained before immunization, after two immunizations and after four immunizations. Two days after each delivery of cyclophosphamide, PBMC and serum were collected. Serum was cryopreserved and PBMC were frozen until use in liquid nitrogen.
Three weeks after the second and fourth immunizations, a 6 mm skin biopsy was obtained from the most recent immunization site to determine the presence of p53-specific T-cells. Biopsy tissue was manually cut into small pieces and infiltrating lymphocytes were expanded by homeostatic proliferation according to SOPs as reported previously [12;25].

Antigens used in immunological assays

Vaccine peptides were divided into four pools: p1-2 (aa 70-115), p3-4 (aa 102-155), p5-7 (aa 142-203) and p8-10 (aa 190-248). Memory recall mix (MRM), a mixture of tetanus toxoid (0.75 limus flocculentius/mL; Netherlands Vaccin Institute, Bilthoven, the Netherlands), tuberculine PPD (0.4 μg/mL; Netherlands Vaccin Institute, Bilthoven, the Netherlands) and *C. Albicans* (0.015% Greenlabs, Lenoir) was used as a positive control.

IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay optimized to measure p53-specific T-cell responses was performed according to SOPs as extensively described previously [12;25-27]. Cells were seeded in quadruplicates at 10^5 cells per well. Responses were considered p53-specific if [(mean number of spots in experimental wells) – (mean number of spots in medium + 2 x SD)] ≥ 10 spots / 10^5 PBMC. A response was considered to be vaccine-induced when (1) the p53-specific response exceeded the pre-existing immune response at least three-fold [26], or (2) when a p53-specific response could be detected in a patient without a p53-specific response prior to immunization.

Proliferation assays

To evaluate proliferative capacity of freshly isolated PBMC in response to stimulation with vaccine and non-vaccine peptides, a 6-day lymphocyte stimulation test (LST) was performed according to SOPs as previously described [12;26]. Skin-biopsy derived lymphocytes were analyzed for the presence of p53-specific T-cells by stimulation with autologous monocytes pulsed overnight with the indicated vaccine peptides in a standard 3-day proliferation assay [12]. Supernatants were cryopreserved. A proliferative response was considered p53-specific when corrected counts per minute (corrected cpm) ≥ 0 (corrected cpm = [(mean ^{3}H-thymidine incorporation) – (mean ^{3}H-thymidine incorporation in medium control + 3xSD ^{3}H-thymidine incorporation in medium control)]) and stimulation index (SI) ≥ 3 (SI = (mean of p53-induced proliferation) / (mean of medium control)) [28]. A vaccine-induced response was defined as (1) a p53-specific response with at least a twofold

increase in corrected counts per minute compared to pre-immunization levels, or (2) a p53-specific response in a patient without p53-specific response prior to immunization.

Cytokine bead array

To evaluate production of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α we analyzed supernatants of proliferation assays by cytokine bead array (LINCO*plex kit*, Linco Research, St. Charles, MO) as described earlier [12;25]. P53-specific cytokine production was defined as concentration of cytokine minus concentration of medium control ≥ 100 pg/mL (IFN-γ), or ≥ 10 pg/mL (other cytokines) and concentration ≥ 2 times medium control. A vaccine-induced response was defined as a p53-specific post-immunization concentration ≥ 2 pre-immunization concentration.

Flow cytometry

PBMC and skin biopsy derived lymphocytes both were evaluated for CD3, CD4, CD8, CD19, CD25, CD56 (IQ Products, Groningen, the Netherlands) and FoxP3 (eBioscience, San Diego, CA) by flow cytometry (FACSCalibur from BD Biosciences, Erembodegem, Belgium) according to manufacturer's instructions.

Treg suppression assay

To evaluate Treg function we used the 'Treg Suppression Inspector' (Miltenyi Biotec, Utrecht, The Netherlands) designed for the functional characterization of human $CD4^+CD25^+$ T-cells by in vitro suppression assays [29;30]. $CD4^+CD25^-$ responder T-cells were co-cultured with $CD4^+CD25^+$ T-cells, isolated with the 'CD4+CD25+ Regulatory T Cell Isolation Kit' (Miltenyi) according to manufacturer's instructions, in three different responder cell: suppressor cell ratios (R:S ratio) (1: 0.1 (10^5:10^4); 1:0.2; 1:0.3, respectively). For T-cell stimulation of both the $CD4^+CD25^-$ responder T-cells and the $CD4^+CD25^+$ T-cells, the Treg Suppression Inspector (CD2, CD3, and CD28 antibodies) was added to the culture. As control, $CD4^+CD25^-$ responder T-cells alone were cultured without any stimulus. Proliferation of T-cells was determined by measuring ^{3}H-thymidine incorporation. Suppression is evaluated by the percentage of maximal proliferation which was calculated as (mean suppressed responder cells (multiple R:S ratio's)) / (mean responder cells) x 100%. The isolated $CD4^+CD25^+$ T-cells were analyzed for FoxP3 positivity, if sufficient material was available for analysis.

(2) CLINICAL RESPONSE

Clinical responses were monitored by serum CA-125 measurements at screening, before each delivery of cyclophosphamide and three weeks after the last immunization, combined with computerized tomography at screening and after study completion. Clinical activity was evaluated according to GCIG criteria [31] by combining serum CA-125 levels with computerized tomography (CT) performed 6-9 weeks after the last immunization and evaluated according to RECIST criteria [32]. Clinically responding patients are defined as patients with either stable disease, partial or complete clinical response after treatment.

(3) SAFETY

For the evaluation of safety, severity of adverse events was graded according to the Common Terminology Criteria (CTC) for Adverse Events v3.0 [33]. Relationship to the p53-SLP vaccine and/or cyclophosphamide was evaluated for all adverse events. A full blood count with differential and serum biochemistry was obtained prior to each gift of cyclophosphamide. The clinical monitoring was similar to our previous study [12].

STATISTICAL ANALYSIS

The number of patients needed to achieve sufficient power to exclude the absence of a clinical response (one-proportion test with alpha level 5%, one-sided) was calculated as 19. Furthermore, with 19 patients included in the study, the upper limit of the 95% exact confidence interval of a true response rate in the absence of clinical responses in a trial is less than 15%, which is the clinical response rate of most drugs registered for the treatment of recurrent ovarian cancer [34]. Unfortunately, due to the outcome of the interim analysis the study stopped at inclusion of 10 patients. Therefore, we were unable to determine our secondary objective clinical efficacy. Differences between pre- and post-immunization were tested for normality by the Shapiro-Wilk test. Normally distributed data ($p \geq 0.05$) were evaluated using a T-test for paired comparisons, and when normality was rejected ($p < 0.05$) a Wilcoxon's signed-ranks test was used. The remainder of tests was similar to the tests used in our previous study [12]. Statistical significance was defined as $p < 0.05$. Statistical Analysis System version 9.1 (SAS Institute, Cary, NC) was used for all analyses.

RESULTS

PATIENT CHARACTERISTICS

Informed consent was obtained from 12 patients. One patient (P106), failed screening because of rapid progressive disease (Fig. 1 in the Supplementary Appendix). Consent was withdrawn for personal reasons by one patient (P104) after receiving a single immunization preceded by cyclophosphamide. According to protocol this patient was used for the safety evaluation only. Two patients (P101, P108) withdrew prematurely from the study because of progressive disease after three and four immunizations combined with cyclophosphamide, respectively. These patients were evaluable for both safety and efficacy analyses consistent with our protocol. All other patients (n = 8) successfully completed the study. Overall, 10 patients were qualified for clinical and immunological analysis. P53-overexpression in the primary tumor was demonstrated by immunohistochemical staining in 45.5% (5/11) of patients (Table 1).

P53-SPECIFIC T-CELL RESPONSES ANALYZED BY IFN-γ ELISPOT

Prior to immunization, responses against vaccine peptides were present in patients P108 (26 specific spots/10^5 PBMC (p1-2)), P111 (62, 90, and 44 specific spots/10^5 PBMC (p1-2, p3-4, and p5-7), respectively) and P112 (20 specific spots/10^5 PBMC (p3-4)). None of these pre-existing p53-specific responses were boosted by the vaccine (Table 1a in the Supplementary Appendix). Vaccine-induced IFN-γ producing p53-specific T-cells however, could be detected in 90% (9/10) of patients after two immunizations (Table 1a in the Supplementary Appendix), and in 87.5% (7/8) of the evaluable patients who received all four immunizations. The strength of the vaccine-induced p53-specific response was robustly enhanced to multiple epitopes within the vaccine as demonstrated by the enhanced reactivity against p1-2 (p = 0.039), p3-4 (p = 0.002), p5-7 (p = 0.004), and p8-10 (p = 0.001) after two immunizations. The p53-specific response to p1-2 was the weakest reaction (Figure 1a). After four immunizations, the response against peptide pools p5-7 and p8-10 (p = 0.039 and p = 0.010, respectively) was significantly higher than the pre-immunization levels. The number of circulating IFN-γ producing p53-specific T-cells remained relatively stable throughout immunizations as no difference in strength of the response against individual peptide pools was observed after the second and fourth immunization ($p \geq 0.236$).

Comparing pre- to post immunization levels, the responsiveness to the mix of recall antigens (MRM) decreased (p = 0.016) (Table 1a in the Supplementary Appendix). This indicates that changes in p53-reactivity resulted from p53-SLP immunization, and did not reflect a generalized increase in responsiveness of the adaptive immune system.

PROLIFERATION ASSAY ANALYSIS OF P53-SPECIFIC T-CELL RESPONSES IN PBMC

Pre-existing p53-SLP vaccine-peptide specific proliferative responses were detected in patients P103 (SI 4, 18, 12, and 4 against each subsequent peptide pool), P105 (SI 9 (p5-7)), P108 (SI 5

Table 1 Patient characteristics

Patient	Age	FIGO stage	Histology	Grade	Residual disease >2cm[1]	Prior chemotherapy	CT-scan at inclusion[2]	P53-overexpression primary tumor[3]
P101	61	IIIc	Serous	3	Yes	First line	ED	+
P102	60	IIIc	Serous	3	No	First line	NED	+
P103	46	IIIb	Serous	1	No	Second line	ED	-
P104	63	IIIc	Serous	3	Yes	First line	Not evaluated	-
P105	60	IIIc	Endometroid	2	No	Fourth line	ED	-
P106	76	III/IV	Serous	3	No	First line	Not evaluated	Not evaluated
P107	61	IV	Serous	Unknown	No	First line	ED	-
P108	55	IIIc	Serous	2	Yes	First line	ED	-
P109	73	IIIc	Serous	2	No	Second line	ED	+
P110	58	IIIc	Serous	2	No	First line	ED	+
P111	59	IIIc	Serous	3	No	First line	ED	+
P112	62	IIIc	Serous	3	No	First line	ED	-

FIGO: International Federation of Gynecology and Obstetrics

[1]Residual disease after primary surgery. [2]ED: evidence of disease; NED: no evidence of disease. [3]p53 expression in the primary tumor analyzed by immunohistochemistry using the p53-specific antibody BP53-12-1.

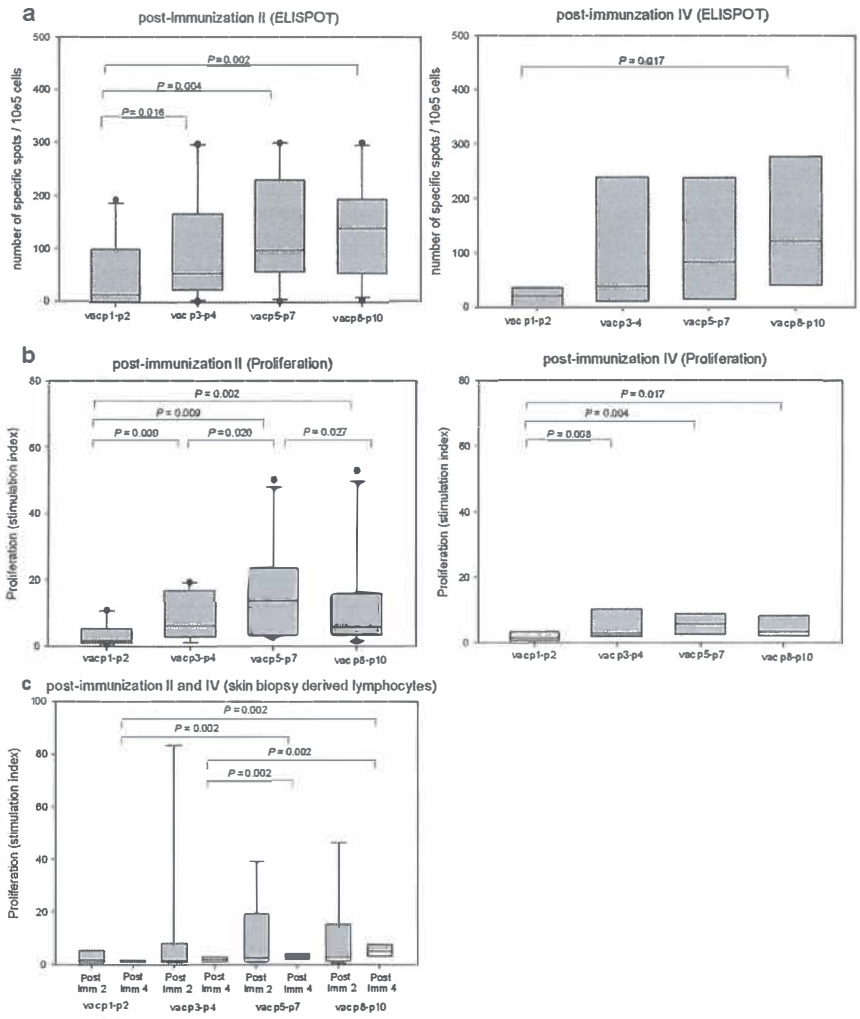


Figure 1 P53-specific responses in ovarian cancer patients immunized with the p53-SLP vaccine preceded by cyclophosphamide. (a) Boxplots comparing responses to vaccine peptide pools as analyzed by IFN-γ ELISPOT using PBMC after II and IV immunizations, in $n = 10$ and $n = 8$ patients, respectively. The number of p53-specific IFN-γ producing cells (per 10^5 PBMC) was calculated by subtracting the mean number of spots + 2xSD of the medium from the mean number of spots of the experimental wells (vertical axis). (b) Boxplots comparing responses to vaccine peptide pools as analyzed by LST using PBMC after II and IV immunizations and (c) skin biopsy derived lymphocytes from the second and fourth injection site of $n = 9$ and $n = 7$ patients, respectively. A proliferative response was considered to be p53-specific when [(mean ^{3}H-thymidine) – (mean ^{3}H-thymidine medium + 3SD ^{3}H-thymidine medium) >0 combined with a stimulation index of ≥3 (SI = mean ^{3}H-thymidine /mean ^{3}H-thymidine medium)] compared to pre-immunization.

(p5-7)), and P111 (SI 5, and 3 (p5-7, and p8-10), respectively). The pre-existing responses in patients P103 (p1-2, p5-7 and p8-10) and P108 (p5-7) were boosted upon two immunizations (Table 1b in the Supplementary Appendix). The remainder of pre-existing responses was not boosted. After two immunizations, vaccine-induced p53-specific responses against vaccine peptides were observed in 80.0% (8/10) of patients, and after four immunizations in 62.5% (5/8) of patients (Table 1b in the Supplementary Appendix).

Similar to the results of the IFN-γ ELISPOT the strength of the vaccine-induced p53-specific response was robustly enhanced after two immunizations in comparison to pre-immunization levels (p1-2: $p = 0.045$; p3-4: $p = 0.004$; p5-7: $p = 0.006$; p8-10: $p = 0.004$). The strength of the p53-specific proliferative response against peptide pools p3-4, p5-7, and p8-10 was higher than responses against peptide pool p1-2 ($p \leq 0.017$). The highest responses were detected against p5-7 (Figure 1b). Compared to pre-immunization, the response after four immunizations only was increased against peptide pool p8-10 ($p = 0.022$).

The proliferative capacity of p53-specific T-cells after four immunizations was a bit weaker than after two vaccinations albeit that this difference was not statistically different when the proliferation against individual peptide pools was compared between the second and fourth immunization ($p \geq 0.068$). The proliferative responsiveness to MRM remained stable over time ($p = 0.994$) (Table 1b in the Supplementary Appendix).

T-HELPER 1 AND T-HELPER 2 CYTOKINE CHARACTERISATION BY CYTOKINE BEAD ARRAY

P53-specific proliferation of PBMC coincided with the production of both Th1 and Th2 cytokines (Figure 2). Th1 cytokines (median [range] IL-2 1.9 [0.0-3.9]; IFN-γ 40.6 [0.0-190.5] pg/ml; TNF-α

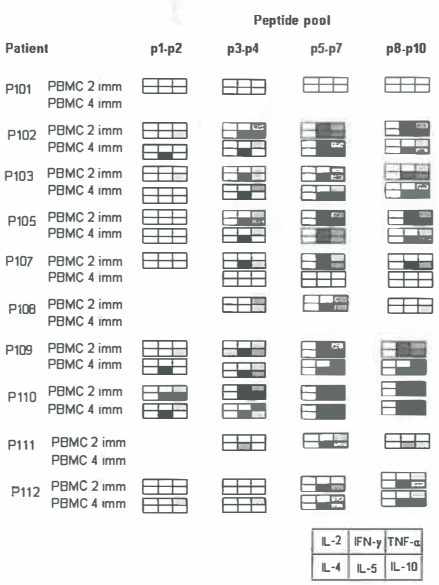


Figure 2 Cytokine profiling of the study cohort. Th1/Th2 cytokine production in PBMC after two and four immunizations with the p53-SLP vaccine preceded by low-dose cyclophosphamide. Cytokines were measured in supernatants produced in proliferation assay. All material analyzed by cytokine bead array is represented by a box. Medium corrected positive production of a cytokine is indicated by a filling (white: <2x medium value; yellow: 2-5x medium; orange: 5-15x medium; red: 15-50x medium; and dark red: >50x higher than medium).

250.9 [0.0-747.2] pg/ml) were detected in p53 peptide-stimulated PBMC isolated after four immunizations. The same holds true for Th2 cytokines (IL-4 1.7 [0.0-2.6] pg/ml; IL-5 272.8 [0.0-495.3] pg/ml; IL-10 46.1 [23.6-124.3] pg/ml). The cytokine levels increased after the second immunization for IFN-γ, IL-10, IL-4 and IL-5 ($p = 0.016$; $p = 0.009$; $p = 0.043$; $p = 0.016$). After four immunizations the cytokine levels for IFN-γ and IL-10 ($p = 0.016$ and $p = 0.014$, respectively) decreased when compared to those measured after two immunizations, but remained higher than pre-immunization levels.

FACS ANALYSIS OF MAJOR LYMPHOCYTE SUBPOPULATIONS IN PBMC

$CD3^+$, $CD4^+$ and $CD8^+$ T-cells increased throughout immunizations ($CD3^+$: $p \leq 0.017$, $CD4^+$: $p \leq 0.022$, $CD8^+$: $p = 0.020$) in comparison to pre-immunization levels, and $CD19^+$ and $CD56^+$ lymphocytes decreased after immunizations ($CD19^+$: $p \leq 0.008$, $CD56^+$: $p \leq 0.021$), compared to pre-immunization levels (Fig. 2 in the Supplementary Appendix). The increase of $CD3^+$ and $CD4^+$ cells throughout immunizations was not observed in all analyzed patients but was predominantly observed in patients P101, P111, and P112 who had an increase of over 25% (data not shown). No change in the percentage of $CD4^+FoxP3^+$ T-cells were observed comparing PBMC at pre-immunization and after each subsequent delivery of cyclophosphamide ($p \geq 0.118$) (Figure 3a).

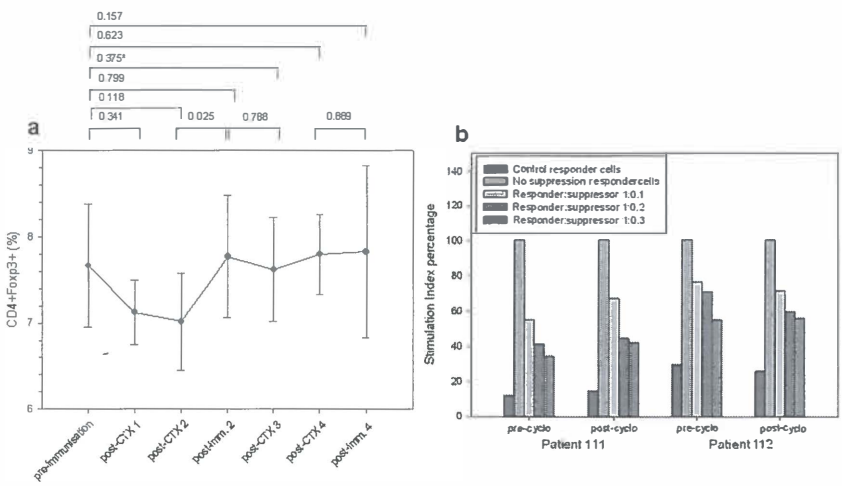


Figure 3 Treg analyses. (a) Evaluation of $CD4^+FoxP3^+$ T cells by flow cytometry of PBMC collected pre-immunization, and after each subsequent delivery of cyclophosphamide. ((b) Evaluating Treg function $CD4^+CD25^-$ responder T-cells were co-cultured with $CD4^+CD25^+$ suppressor Tregs in three different responder cells: suppressor cells ratios (1: 0.1 (10^5:10^4); 1:0.2; 1:0.3, respectively) with T-cell stimulation added to the culture. Suppression was calculated as % of maximal proliferation as (mean suppressed responder cells (multiple R:S ratio's)) / (mean responder cells) x 100%. Responder cells were also measured without suppression, with T-cell stimulation only. Responder cells were measured without T-cell stimulation, as depicted in this figure.

QUALITATIVE ANALYSIS OF REGULATORY T-CELLS

To evaluate Treg function we performed a qualitative analysis using the PBMC isolated before and three weeks after the last dose of cyclophosphamide from patients P111 and P112. The purity of $CD4^+CD25^-$ T-cells and $CD4^+CD25^+$ T-cells analyzed was 97% in both cases, as assessed by flow cytometry. In the samples analyzed, $CD4^+CD25^+$ T-cells indeed were FoxP3 positive. We observed a decreased percentage of maximal proliferation of 70%, 50%, and 40%, respectively with increasing R:S ratios (Figure 3b). We observed no difference in percentage of maximal proliferation before treatment or after four immunizations preceded by four cycles of cyclophosphamide.

P53-SPECIFIC T-CELL MIGRATION IN THE SKIN ANALYZED BY PROLIFERATION ASSAYS

To analyze the migratory capacity of p53-specific T-cells to sites where p53 antigen is presented, we cultured lymphocytes from skin biopsies taken from the second and fourth injection sites (n = 9 and n = 7, respectively). After four immunizations a difference in proliferating T-cells is observed between different peptide pools, i.e. responses against p5-7 and p8-10 are higher than p1-2 and p3-4 (p = 0.002) (Figure 1c). One patient (P111) displayed very high responses only against peptide pool p3-4 after two immunizations (Figure 1c). Proliferation against p1-2 and p8-10 was more pronounced in biopsies taken from the fourth vaccination site when compared to what was observed in the biopsies from the second vaccine site (p = 0.028). Vaccine-site infiltrating p53-specific T-cells were detected in 44.4% (4/9) of biopsies taken from the second vaccination site and in 85.7% (6/7) of the fourth vaccination site. Phenotyping of the infiltrating lymphocytes from these biopsies by flow cytometry revealed a preponderance of $CD3^+$ T-cells (mean ± SEM: 64.4% ± 9.8 and 73.8% ± not applicable (n = 1), 2^{nd} vs. 4^{th} vaccination site, respectively), of which 52.5% ± 8.5 and 53.5% ± 7.0 were $CD4^+$, 23.0% ± 6.2 and 18.0% ± 2.7 were $CD8^+$, respectively. $CD4^+FoxP3^+$ cells were not detected among the infiltrating lymphocytes.

CLINICAL RESPONSE

Two patients (20%) had stable disease as evaluated by CA-125 and CT-scan (P108, P109). In both patients, vaccine-induced p53-specific responses were present. The other patients (8/10; 80%) had clinical, biochemical and/or radiographic evidence of progressive disease. Seven patients had decreasing or stable CA-125 levels during the study (P101, P103, P107, P108, P109, P110 and P111) (Figure 4a). The strength of the p53-specific responses measured by ELISPOT and LST after two and four immunizations from these 7 patients was not significantly different from the patients showing rising CA-125 levels (p > 0.293) (Figure 4b).

TREATMENT-RELATED TOXICITIES

No vaccine-related CTC grade 3 or 4 adverse events were observed (Table 2 in the Supplementary Appendix). Patient P101 and P108 developed ileus and omental metastases, respectively, due to disease progression for which they were hospitalized. No major vaccine- or cyclophosphamide-

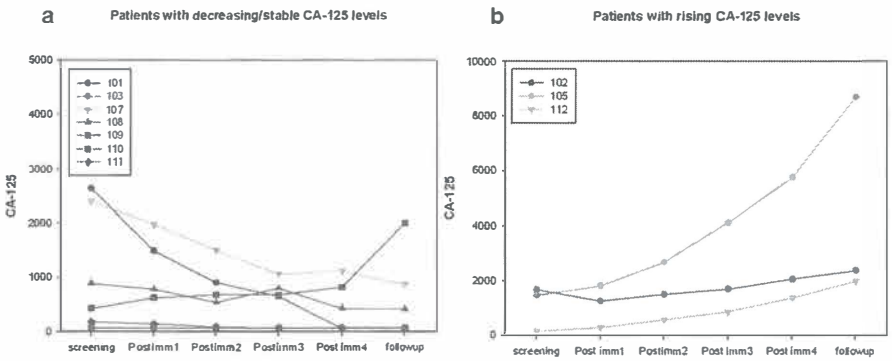


Figure 4 CA-125 curves of individual patients. Evaluation of serum CA-125 levels throughout immunizations preceded by cyclophosphamide as measured at screening, after each subsequent immunization, and at follow-up visits with either (a) decreasing/stable or (b) rising levels during participation in the trial. Each coloured line corresponds to CA-125 levels measured in a single patient. Patient numbers were noted in the graph.

induced alterations were observed in serum biochemistry and complete blood count. Mild to moderate inflammatory symptoms at the injection site occurred in nearly all patients; redness and swelling lasted 15-16 days, and pain and itch 4-5 days on average. (Re)-activation of local inflammatory symptoms at prior injection sites was observed in the majority of patients (90%) after subsequent immunizations. We did not find evidence of vaccine-induced auto-immunity, assessed by clinical symptoms and serum antinuclear antibody levels (data not shown).

RESULTS OF P53-SLP PRECEDED BY CYCLOPHOSPHAMIDE COMPARED TO P53-SLP ALONE

Analyses of the clinicopathological stage, histology, grade, residual disease, prior courses of chemotherapy, evidence of disease at inclusion, and p53-overexpression of primary tumor, revealed no differences between patients treated in the current and the previous study, suggesting that the two different patient groups are similar and to some extent comparable (Table 3 in the Supplementary Appendix).

Notably, analysis of the results obtained by IFN-γ ELISPOT revealed that the reactivity against each individual peptide pool differed between the current and the previous study ($p \leq 0.012$) (Figure 5a). Stronger reactivity was found against all peptide pools after two and four immunizations, except for p1-2 ($p = 0.060$) after two immunizations. In the previous study a strong reduction was observed in the number of circulating IFN-γ producing p53-specific T-cells after four immunizations. This reduction however, was not observed in the current study, rather responses remained stable. Comparison of the cumulative response to peptide pools between the current and previous trial showed that the reactivity, as reflected in median number of cumulative spots, differed between the two trials at two immunizations (peptide pools p1-10 median (IQR) current study: 329 (242-

388); previous study: 70 (42-205), respectively), as well as at four immunizations (peptide pools p1-10 median (IQR) current study: 285 (102-698); previous study: 40 (5-82), respectively) (Figure 5b). No differences were observed between the two trials with respect to the proliferative capacity of p53-specific T-cells as measured by LST (data not shown) or in the amounts of p53-specific proliferation-associated cytokine production (Figure 5c).

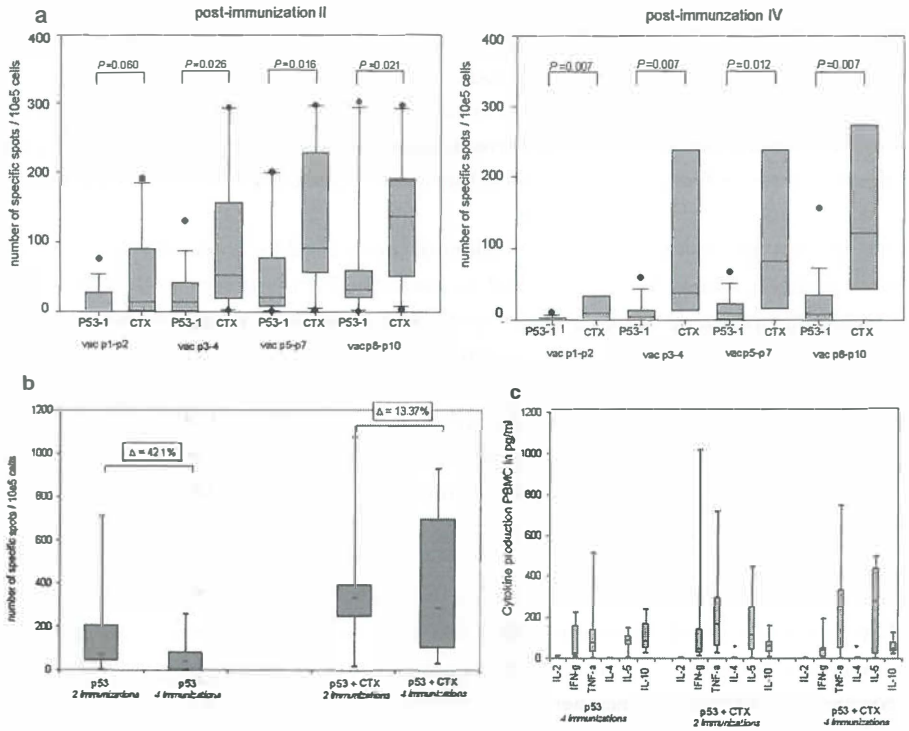


Figure 5 Comparison of p53-SLP preceded by cyclophosphamide and p53-SLP immunization alone [12]. (a) Differences in induction of p53-specific responses determined by ELISPOT after treatment with the p53-SLP vaccine combined with cyclophosphamide (CTX) and the p53-SLP vaccine alone (P53-1) against each individual peptide pool after two and four immunizations. (b) Boxplots represent p53-specific responses determined by ELISPOT in the current and previous study. Difference in median values of stimulation with all peptide pools (p1-10) after two and four immunizations in the previous and current study is depicted in the figure in percentage, as measured in n = 19, n = 18, n = 10 and n = 8 patients, respectively. (c) Box plots represent median cytokine levels measured in supernatants of proliferation assays stimulated with peptide pool p1-p10 analyzed by cytokine bead array after four immunizations with or without addition of cyclophosphamide in n = 7 and n = 8 ovarian cancer patients, respectively. Also cytokine levels induced by two immunizations preceded by cyclophosphamide were depicted, as measured in n = 10 patients. * = significant difference between cytokine levels induced upon immunization combined with cyclophosphamide compared to immunization alone.

DISCUSSION

Combination of low-dose cyclophosphamide with a p53-SLP vaccine induced no quantitative or qualitative reduction of Tregs in 10 advanced stage epithelial ovarian cancer patients. However, we did observe a strong Th1/Th2 immune response to p53 in this phase II single-arm study. This shows that the use of low-dose cyclophosphamide two days prior to each p53-SLP immunization does not impair de novo induction of vaccine-induced p53-specific T-helper cell immunity. Furthermore, the number of vaccine-induced p53-specific IFN-γ producing T-cells was higher in the current study, compared to a study in which a similar patient group was treated with p53-SLP monotherapy. Moreover, the strong reduction in the number of circulating p53-specific T-cells observed previously after four immunizations was absent in the current study. Our results warrant new studies on the use of low-dose cyclophosphamide to potentiate the immunogenicity of anti-tumor vaccines.

Cyclophosphamide in dosages half or less the equivalent of those used for chemotherapy in combination with immunotherapy have been described to decrease Treg numbers and impair their function [20;35]. Cyclophosphamide appears to be most effective when administered several days prior to immunization [36;37]. Contrary to our expectations, we observed that $CD4^+FoxP3^+$ T-cells were not depleted by addition of 300 mg/m^2 i.v. cyclophosphamide two days prior to the p53-SLP vaccine, nor did cyclophosphamide affect the in vitro suppressive capacity of $CD4^+CD25^+$ suppressor cells. These observations are supported by Audia *et al*., who previously reported that cyclophosphamide combined with immunotherapy failed to reduce the frequency of regulatory T-cells or significantly modulate their function [38].

Addition of cyclophosphamide to the p53-SLP vaccine resulted in induction of high numbers of p53-specific IFN-γ^+ producing T-cells against multiple p53 epitopes in the majority of ovarian cancer patients. Induction of tumor-specific immune responses in cancer patients treated with cyclophosphamide combined with specific vaccinations were also reported by others [39;40], suggesting that targeting function and frequency of regulatory T-cells by cyclophosphamide in cancer patients unmasks and potentially enhances tumor-specific T-cell responses. When comparing the results obtained in this trial to the results of our previous trial, we observed that the number of IFN-γ producing T-cells was higher and remained more stable in the group of patients receiving the p53-SLP vaccine combined with cyclophosphamide, suggesting that cyclophosphamide treatment augmented Th1 reactivity. However, no difference was observed in the p53-specific proliferation and associated cytokine production of these proliferating cells implying that rather than the expansion of p53-specific T-cells their in vivo lifespan or function was altered. Recently, Ding *et al.* showed that pre-treatment with cyclophosphamide may augment tumor-specific $CD4^+$ T-cell immunity by rescuing these cells from apoptosis through the prevention of PD-1 up-regulation and IL-7R down-regulation on $CD4^+$ effector T-cells [41]. A similar phenomenon may play a role in our study.

In a clinical case report on NY-ESO-1-specific immunotherapy, it was shown that tumors regressed completely after adoptive transfer of NY-ESO-1-specific $CD4^+$ T-cells, even though NY-ESO-1 was

not uniformly expressed by the tumor cells [42]. This complete regression of tumor was explained by the induction of T-cell responses to two other tumor-associated antigens displayed by the tumor, i.e. MART-1 and MAGE-3, following infusion of NY-ESO-1 specific $CD4^+$ T-cells [42]. This additional response is likely to have been triggered by the NY-ESO-1 $CD4^+$ T-helper cell which induced activation of antigen presenting DC that had ingested autogenic material from cancer cells. We observed that the p53-SLP vaccine strongly activates p53-specific $CD4^+$ T-cells but not $CD8^+$ T-cells. Analogous to the study described above, a strong p53-specific $CD4^+$ T-cell response may trigger the responses of $CD8^+$ T cells to other ovarian cancer expressed antigens, e.g. the highly immunogenic Wilms' tumor protein 1 antigen [43;44].

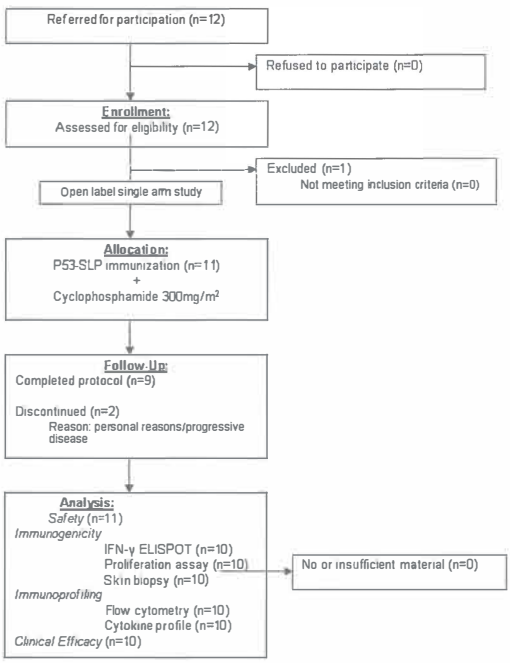
Finally, our results fit in with the safety and immunogenicity experience gathered thus far with vaccines consisting of synthetic long peptides dissolved in Montanide ISA-51 adjuvant, showing only low-grade toxicity and strong immunogenicity. Despite the observed lack of influence on $CD4^+FoxP3^+$ T-cells in this study, cyclophosphamide treatment, at this dose schedule, is a promising immune-potentiating strategy for anti-p53 vaccines. The information gained from this study will serve as a baseline for further clinical investigation to better define the full potential of this strategy to ultimately achieve antitumor immunity with clinical impact. Future studies will be needed to establish possibilities to induce robust anti-tumor immunity by induction of antigen-specific cytotoxic T-lymphocyte alongside p53-SLP induced p53-specific Th cells.

ACKNOWLEDGMENTS

We thank all patients who participated in this study, and the Trial Coordination Center of the UMCG for study monitoring and statistical analyses.

SUPPLEMENTARY APPENDIX

Figure 1 Consort Flowchart



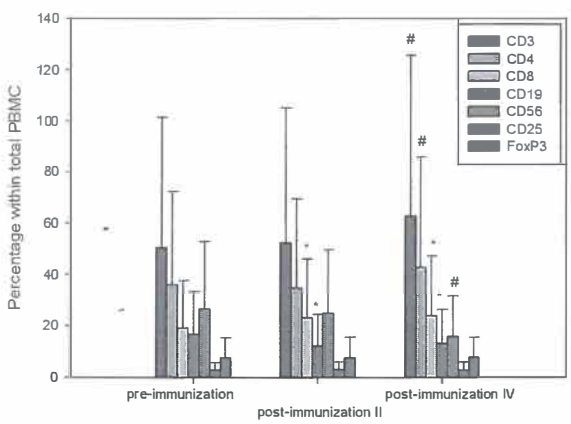


Figure 2 Major lymphocyte subpopulation analysis. Evaluation of CD3, CD4, CD8, CD19, CD56, CD25, and FoxP3 by flow cytometry of PBMC collected pre-immunization, and after two and four immunizations in $n = 10$, $n = 10$ and $n = 8$ patients, respectively. Bars correspond to mean percentage of CD cells, with the standard error of the mean. * A significant difference compared to pre-immunization levels. # A significant difference compared to levels measured at pre-immunization and after two immunizations.

Table 1a Response in PBMC analyzed by IFN-γ ELISPOT

	Before immunizations					After two immunizations					After four immunizations				
Patient	p1-p2	p3-p4	p5-p7	p8-10	MRM	p1-p2	p3-p4	p5-p7	p8-10	MRM	p1-p2	p3-p4	p5-p7	p8-10	**MRM**
P101	4	2	5	1	51	10	5	5	5	32	na	na	na	na	na
P102	2	1	7	4	300	**135**	**300**	**300**	**256**	219	**39**	**300**	**276**	**300**	300
P103	1	3	3	4	300	4	**61**	**40**	**144**	165	**15**	**35**	**140**	**118**	122
P104	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
P105	5	8	8	2	71	2	**72**	**108**	**84**	102	6	**73**	**83**	**137**	25
P106	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
P107	1	2	1	1	16	**19**	**51**	**84**	**58**	5	1	**33**	**33**	**25**	9
P108	32	1	4	2	300	1	**32**	**215**	**142**	87	na	na	na	na	na
P109	126	75	188	69	300	**176**	**156**	**300**	**300**	153	**178**	**87**	**132**	**252**	146
P110	1	2	5	1	25	**194**	**288**	**300**	**300**	4	**30**	**300**	**300**	**300**	10
P111	141	169	122	87	55	186	223	189	**152**	x	285	252	248	213	x
P112	3	22	2	11	105	**15**	50	**66**	**177**	107	2	10	**14**	**98**	39

Responses are depicted as number of spots per 10^5 PBMC. Bold corresponds to vaccine-induced p53-specific responses (see definitions in material and methods). na = PBMC were not available. x = not detectable on well.

Table 1b Response in PBMC analyzed by Lymphocyte Stimulation Test

	Before immunizations					After two immunizations					After four immunizations				
Patient	p1-p2	p3-p4	p5-p7	p8-10	MRM	p1-p2	p3-p4	p5-p7	p8-10	MRM	p1-p2	p3-p4	p5-p7	p8-10	**MRM**
P101	2346	3078	3330	4309	6211	**3862**	**5630**	**4343**	**4530**	15273	*na*	*na*	*na*	*na*	*na*
P102	253	532	377	693	8707	**1359**	**4918**	**7657**	**5010**	3681	**4130**	**20804**	**7568**	**4153**	11438
P103	738	3295	2123	729	14336	**1924**	3471	**5198**	**3663**	14917	592	1779	1437	**1833**	9223
P104	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
P105	239	661	4786	734	3378	535	**2631**	6308	**3855**	4150	624	2299	4228	**3159**	1815
P106	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
P107	754	905	1659	1379	1480	1510	**4380**	**13193**	**3910**	1220	640	**1513**	1296	1383	877
P108	249	513	1480	277	19079	184	**3818**	**3321**	**1599**	4490	*na*	*na*	*na*	*na*	*na*
P109	3875	1968	4391	4313	10216	1689	3067	**5533**	**4294**	7185	1156	**3473**	**6882**	**5157**	10978
P110	191	287	587	237	431	**2720**	**4763**	**14431**	**15206**	490	1220	**2183**	**5029**	**3351**	884
P111	1547	1346	3900	2411	2492	1055	1252	2619	1484	3924	918	2070	3153	2458	2287
P112	137	144	265	188	2807	760	1736	**4002**	3342	5290	273	1483	**2087**	1433	2612

Responses are depicted as counts per minute. Bold corresponds to vaccine-induced p53-specific responses (see definitions in material and methods). *na* = PBMC were not available.

Table 2 Number of adverse events

	Number of events (number of patients)				
CTC grade[1]	1	2	3	4	5
Abdominal neoplasm	-	3 (3)[2]	-	-	-
Abdominal pain	2 (2)[2]	5 (5)[2]	-	-	-
Anaemia	1 (1)	1 (1)	-	-	-
Arthralgia	1 (1)	2 (1)	-	-	-
Ascites	-	2 (2)[2]	-	-	-
Back pain	-	1 (1)	-	-	-
Breast pain	1 (1)	-	-	-	-
Bronchitis	-	-	1 (1)[3]	-	-
Cardiac murmur	1 (1)	-	-	-	-
Circulatory collapse	-	1 (1)	-	-	-
Constipation	2 (2)	1 (1)	-	-	-
Dental discomfort	-	1 (1)	-	-	-
Diarrhea	-	1 (1)	-	-	-
Dizziness	1(1)	-	-	-	-
Dyspnoe	-	1 (1)	-	-	-
Extrapyramidal disorder	1(1)	-	-	-	-
Fatigue	1 (1)	2 (2)	1 (1)[2]	-	-
Flank pain	1 (1)	-	-	-	-
Flu like symptoms	1 (1)	3 (2)	-	-	-
Gastrooesophag. reflux	1 (1)	-	-	-	-
Headache	2 (2)	1 (1)	1 (1)[4]	-	-
Leukopenia	-	1 (1)	-	-	-
Lymphopenia	-	1 (1)	-	-	-
Monocytopenia	-	1 (1)	-	-	-
Muscle spasms	1 (1)	-	-	-	-
Musculoskeletal pain	-	1 (1)	-	-	-
Nasopharyngitis	-	1 (1)	-	-	-
Nausea	11 (8)[5]	19 (5)[5]	-	-	-
Neutropenia	-	1 (1)	-	-	-
Oedema peripheral	2 (1)	1 (1)	-	-	-
Palpitations	1 (1)	-	-	-	-
Proctalgia	-	1 (1)	-	-	-
Pyrexia	4 (3)	-	-	-	-
Rash	-	1 (1)	-	-	-
Systolic hypertension	1 (1)	-	-	-	-
Tooth extraction	1 (1)	-	-	-	-
Vomiting	3 (2)[5]	4 (3)[5]	-	-	-
Weight loss	1 (1)[2]	1 (1)[2]	-	-	-

[1]Adverse events are graded according to CTC criteria v3.0. [2]Related to disease progression [3]Developed bronchitis after first gift of cyclophosphamide. [4]Pre-existent illness. [5]Related to administration of cyclophosphamide.

Table 3 Clinicopathological characteristics p53-SLP treated patients with or without CTX

	P53-SLP alone	P53-SLP with CTX	
	n (%)	n (%)	*p*
FIGO[1] *stage*			
Early[2] stage	2 (10.0)	0 (0.0)	0.258
Advanced[3] stage	18 (90.0)	12 (100.0)	
Tumor type			
Serous	13 (65.0)	11 (91.7)	0.092
Non-serous[4]	7 (35.0)	1 (8.3)	
Tumor grade			
Low grade	7 (35.0)	5 (45.5)	0.567
High grade	13 (65.0)	6 (54.5)	
Residual disease[5]			
Absent	11 (64.7)	9 (75.0)	0.555
Present	6 (35.3)	3 (25.0)	
Prior chemotherapy			
First line	12 (60.0)	9 (75.0)	0.333
More courses	8 (40.0)	3 (25.0)	
Evidence disease at inclusion[6]			
NED	5 (25.0)	1 (10.0)	0.981
ED	15 (75.0)	9 (90.0)	
P53-overexpression primary tumor[7]			
Normal expression	11 (55.0)	6 (54.5)	0.387
Overexpression	9 (45.0)	5 (45.5)	

[1]FIGO = International Federation of Gynaecology and Obstetrics. [2]Early stage: stage I and II. [3]Advanced stage: stage III and IV.. [4]Non-serous: mucinous, endometrioid, clear cell and undifferentiated epithelial ovarian cancer. [5]Residual disease after primary surgery. [6]NED = no evidence of disease; ED = evidence of disease. [7]p53 expression in the primary tumor analyzed by immunohistochemistry using the p53-specific antibody BP53-12-1.

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CHAPTER 4

IMMUNOLOGICAL AND CLINICAL EFFECTS OF VACCINES TARGETING P53-OVEREXPRESSING MALIGNANCIES

REVIEW ARTICLE

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ABSTRACT

Approximately 50% of human malignancies carry p53 mutations, which makes it a potential antigenic target for cancer immunotherapy. Adoptive transfer with p53-specific cytotoxic T-lymphocytes (CTL) and $CD4^+$ T-helper cells eradicates p53-overexpressing tumors in mice. Furthermore, p53 antibodies and p53-specific CTLs can be detected in cancer patients, indicating p53 is immunogenic. Based on these results clinical trials were initiated. In this article we review immunological and clinical responses observed in cancer patients vaccinated with p53 targeting vaccines. In the second part of this review, we summarize several immunopotentiating combination strategies suitable for clinical use.

In most trials p53-specific vaccine-induced immunological responses were observed. Unfortunately, no clinical responses with significant reduction of tumor-burden have occurred. We will elaborate on possible explanations for this lack of clinical effectiveness.

In our opinion, future p53-vaccine studies should focus on addition of these immunopotentiating regimens to achieve clinically effective therapeutic vaccination strategies for cancer patients.

1. INTRODUCTION

Despite recent progress in surgical, chemotherapeutic, and radiotherapeutic approaches, cancer is still difficult to treat and cure, especially in patients with advanced stage of disease. Therefore, new therapeutic strategies are required. One of the new treatment strategies is immunotherapy targeting tumor-associated antigens (TAA).

Mutation of the p53 tumor-suppressor gene is a frequent event in human oncogenesis. The role of the p53 gene has been reviewed extensively by Vogelstein and Vousden [1-3]. P53 mutations found in tumors were shown to abrogate the regulatory function of p53 on the cell cycle. Moreover, many mutations lead to an increased half-life of the otherwise rapidly degraded p53 protein and thereby to accumulation of this protein in cells [4]. Other tumor suppressor genes often lose their expression after mutation, but the point mutated p53 protein is often more stable and therefore overexpressed in tumor cells [5;6]. P53 degradation can also be promoted directly through binding to viral proteins or deletions promoting presentation for T cell recognition [1;2].

$CD8^+$ cytotoxic T-lymphocytes (CTLs) are the most important effector cells for antitumor immune responses. They recognize TAA-derived peptides that are processed and presented on the tumor cell surface in association with major histocompatibility complex (MHC) class I molecules, leading to killing of tumor cells [7]. Processing of the intracellular p53 protein by the proteasome will result in presentation of p53-derived peptides in the context of MHC class I molecules at the tumor cell surface. $CD4^+$ T-helper (Th) cells play an important role in orchestrating and sustaining the local immune attack by CTL [8;9]. In contrast, $CD4^+$ $FoxP3^+$ regulatory T-cells (Tregs) impede antitumor immunity by inhibiting CTL activation [10;11].

The search for widely expressed tumor antigens as targets for MHC class I restricted CTLs is of great importance for the development of T cell-mediated immunotherapy of cancer. As persistent overexpression of p53 or induced T cell presentation is present in ~ 50% of a wide variety of cancers, a large group of patients would benefit from p53 directed immunotherapy.

Since p53 is a self-antigen expressed at low levels in normal cells, immunogenic tolerance might hinder the use of wild type p53 as a tumor antigen for immunotherapeutic approaches. Moreover, the idea of targeting a non-mutated wild type p53 gene with a vaccine may be counterintuitive. So far induction of p53-specific CTL and Th-cells with the capacity to eradicate p53-presenting tumors without inducing clinical nor immunopathological damage to normal tissue has been observed in different mouse models, despite the fact that wild type p53 is expressed in normal tissue [12-14]. This tumor selectivity could be explained by the increased p53 protein expression resulting from p53 mutation [13]. Alternatively, insufficient antigen display in normal tissues by the MHC class I molecule in combination with lack of or proper costimulation and downregulatory chemokine and cytokine conditions might protect against the destruction by the potentially autoreactive wild type p53-specific CTL [15;16]. Consequently, wild-type p53-specific CTls are able to discriminate between p53-presenting tumor cells and normal tissue, indicating the that widely expressed autologous molecules such as p53 can serve as a target for CTL-mediated immunotherapy of tumors [17]. In humans, spontaneous MHC class I restricted p53-specific CTL [18;19], MHC class II restricted p53-specific proliferating Th-cells [20;21], and p53 antibody responses have been observed

[22;23]. Furthermore, several naturally processed human wild-type p53 derived epitopes in both MHC class I and MHC class II have been identified [17]. The presence of cellular and humoral immune responses against p53 shows that tolerance is not complete for this self antigen. In particular CD4 T cell tolerance, based on mouse observations, is far from profound [24].

On the basis of these preclinical results, which indicate the occurrence of p53-directed immune responses in cancer patients, several clinical trials have been performed with vaccines targeting p53. These studies have, however, generally not yet evolved past phase I/II studies.

In this review the immunogenicity and clinical efficacy of p53-specific active immunotherapy in human cancer is evaluated to assess the potential of this treatment modality for cancer. Furthermore, we propose a few straightforward clinically applicable combination strategies to improve clinical efficacy of p53-directed immunotherapies.

2. CLINICAL TRIALS OF P53 PEPTIDE CANCER VACCINE

Several phase I/II immunization trials using p53 immunogens have been conducted so far (Table 1). We have summarized the observed immune and clinical responses in cancer patients, induced by the p53-vaccine (Table 2). Next, we provide a more detailed account of the studies, categorized by the different vaccination strategies.

VIRAL VECTOR-BASED VACCINES

Viral vectors encoding recombinant transgenes for TAAs (such as p53) capable of infecting host cells, can elicit a tumor-specific immune response against the transgene product. Recombinant viral vector-vaccines encoding full-length TAA may contain epitopes for both $CD4^+$ T-helper (Th) cells and $CD8^+$ cytotoxic T-lymphocytes (CTLs). The clinical advantage of this vaccination strategy therefore is that the MHC type of the individual patient does not need to be considered (reviewed in [25-28]). Several clinical studies on viral vector-based vaccines encoding p53 have been conducted.
In a pilot study, Kuball *et al.* immunized six advanced stage cancer patients with a recombinant replication-defective adenoviral vector encoding human full-length wild-type p53 [29]. Neither tumor responses nor anti-p53 responses were observed, however all patients showed an adenoviral immune response. This strong anti adenoviral-specific response may have competed out the p53-specific response. Clinical tumor responses were assessed by imaging diagnostics using National Cancer Institute response criteria. Three months after initial immunization, 4 patients had stable disease. After follow-up of 7-16 months only one patient had stable disease.
Based on preclinical results in mice and rhesus macaques, Menon *et al.* performed a phase I/II clinical study involving vaccination of end-stage colorectal cancer patients with a recombinant canarypox virus (ALVAC) encoding wild type p53 [30;31]. Patients were immunized intravenously with an increasing dosage of ALVAC-p53. From this study it appeared that this modality is safe and capable of stimulating p53-specific Th1 (IFN-γ) responses in several of these patients. One out of 16 patients showed stable disease for a short period of time after immunization with the highest dose. Fever was the only vaccine related adverse event. The authors conclude from this trial that repeated immunizations are probably necessary to obtain good clinical responses. Again, anti-vector responses were observed in all patients after vaccination which, by antigenic competition, may have prevented robust anti-p53 immune responses.
In a phase I/II study Antonia *et al.* tested a cancer vaccine consisting of dendritic cells transduced with the full-length wild-type p53 gene delivered via an adenoviral vector [32]. Significant p53-specific T cell responses to vaccination were found in 13 out of 25 patients (52%) in IFN-γ ELISPOT assays. In 7 out of 12 HLA-A2 positive patients, an increase in frequency of $CD8^+$ T cells that secrete IFN-γ in response to targets pulsed with an HLA-A2 restricted p53 peptide were found. Four out of 10 patients with a detectable pre-immunization level of anti-p53 antibody developed a positive p53-specific T cell response to vaccination. No link was found between the presence of $CD4^+$ $FoxP3^+$ regulatory T-cells (Tregs) and p53-specific T-cell responses to vaccination in the patient's blood be-

Table 1 P53-targeting vaccines in human cancer

Author	Year	Study	Vaccine	Tumor site	n	Disease status	Previous treatment	Imm*	Ref
Kuball et *al*	2002	Pilot study	recombinant virus	urogenital-, lung cancer, malignant schwannoma	6	advanced disease	unknown	4	[29]
Menon et *al.*	2003	Phase I/II	recombinant virus	colorectal cancer	16	metastatic disease	chemotherapy/radiation therapy/ other	3	[30; 31]
Antonia et *al.*	2006	Phase I/II	recombinant virus	small cell lung cancer	29	extensive/ recurrent disease	chemotherapy (1 to ≥3 regimens)	±3	[32]
Svane et *al.*	2004	Phase I	peptide pulsed DC	breast cancer	6	progressive/ metastatic disease	chemotherapy/ radiotherapy/endocrine therapy	10	[41]
Svane et *al.*	2007	Phase II	peptide pulsed DC	breast cancer	26	progressive/ metastatic disease	chemotherapy (1-5 regimens)/endocrine treatment (1-3 regimens)	10	[42]
Lomas et *al.*	2004	Phase I	short peptide	breast, colorectal, non-small-cell lung, renal, prostate, head- and neck, hemangio-pericytoma, esophageal cancer	14	NED/ metastatic / recurrent disease	yes	4	[47]
Rahma et *al.*	2010	Phase II	short peptide/ peptide pulsed DC	ovarian cancer	21	NED	surgery/chemotherapy	≤31	[48-50]
Leffers et *al.*	2009	Phase II	long peptides	ovarian cancer	20	recurrent disease	surgery/chemotherapy	4	[52]
Speetjens et *al.*	2009	Phase I/II	long peptides	colorectal cancer	10	metastatic disease	surgery/chemotherapy	2	[53]

NED: no evidence of disease.*Number of immunizations

Table 2 Immune and clinical response p53-targeting vaccines

Author	Year	Humoral response[1]	Cellular response[2]		Immunohistochemistry[3]	Clinical response[4]	Toxicity	Ref
			ELISPOT	Proliferation				
Kuball *et al*	2002	no anti-p53 specific Abs	no p53-specific response	not analyzed	3/6 positive	4/6 SD 2/6 PD	CTC I, local reaction, fever	[29]
Menon *et al.*	2003	pre 7/15 post 10/15	4/15 PR	1/15 PR	not analyzed	1/16 SD	CTC I/II, fever	[30; 31]
Antonia *et al.*	2006	pre 10/22 post 10/22	16/28 PR	p53-specific proliferation not analyzed	not analyzed	1/29 PR* 7/29 SD* 21/29 PD*	CTC I/II	[32]
Svane *et al.*	2004	not analyzed	4/6 PR	not analyzed	3/6 positive	2/6 SD* 2/6 PD* 2/6 MR/UR*	mild/moderate local reaction/flu-like symptoms	[41]
Svane *et al.*	2007	not analyzed	8/22 PR	not analyzed	11/26 positive	8/19 SD* 11/19 PD*	CTC I/II, local reaction, flu-like symptoms	[42]
Lomas *et al.*	2004	pre 0/6 post 1/6	0/6 PR	2/6 VIR	14/14 positive	Not analyzed	CTC I/II, local reaction, nausea, arthralgia	[47]
Rahma *et al.*	2010	not analyzed	10/19 PR	not analyzed	21/21	3 NED 1 SD 16 PD	CTC III/IV	[48-50]
Leffers *et al.*	2009	pre 8/20 post 9/20	18/18 PR	14/17 PR	9/20 positive	2 SD* 18 PD*	CTC I/II, local reaction	[52]
Speetjens *et al.*	2009	not analyzed	6/9 PR	7/10 VIR	6/10 positive	3/10 NED 7/10 PD	CTC I/II, local reaction, flu-like symptoms	[53]

[1]pre- and postimmunization levels of anti-p53 specific antibodies. [2]p53-specific T-lymphocytes induced by immunizations, PR: positive response, VIR: vaccine induced response. [3]p53- staining of primary tumor samples. [4]SD: stable disease, PD: progressive disease, MR: mixed response, UR: unconfirmed regression, PR: partial response, NED: no evidence of disease, *all according to Response Evaluation Criteria in Solid Tumors.

fore or after vaccination, despite the assumption that Tregs downregulate the antitumor immune response. Objective clinical responses were observed in 61.9% of 21 patients treated with second-line chemotherapy directly after immunization. This result provides direct clinical evidence that cancer vaccines may be most effective not as a single modality, but rather in close combination with other methods of treatment, specifically, chemotherapy. This observed effect could be explained by a number of potential mechanisms, such as down-regulation of the effect of tumor-produced immunosuppressive factors that prevent CTLs from killing tumor cells by chemotherapy [33], or up-regulation of p53 in tumor cells, which can make them more susceptible to recognition by CTLs [34], or lastly, chemotherapy may make tumor cells more susceptible to the cytotoxic effect of CTLs through a perforin-independent increase in permeability to granzyme B released by the CTLs [35]. Collectively, viral vector-based vaccines encoding p53 are well tolerated in early-phase clinical trials with minimal toxicity. Limited p53-specific immune responses might be due to antigen competition, as all patients had strong anti-vector responses. Future studies on viral vector-based vaccines should focus on the use of prime-boost strategies with different vectors delivering p53. This strategy overcomes the antigenic competition in priming with viral vectors. Viral vector recombinant Semliki Forest virus which is not strongly affected by vector-neutralising antibodies therefore, has exquisite potency in homologous prime-boost immunization regimens [36].

DENDRITIC CELL-BASED VACCINES

It is important to investigate the character of the p53 specific T-cell responses, because p53-based vaccination of patients should be aimed at boosting only the desired Th1-type immunity, while stimulation of Th2-type or Tregs should be avoided [37]. This finding would argue in favour of application of a p53-specific vaccination using a delivery mode specifically stimulating the anti p53 (CTL) and Th1 responses. Autologous dendritic cells (DC) expressing the antigen of interest could be one of these ways (reviewed in [38-40]). Dendritic cells are highly potent professional antigen-presenting cells (APCs). Therefore, antitumor vaccines have been designed, using DCs generated on clinical scale loaded with synthetic MHC binding peptides known to stimulate peptide specific CTLs, like p53.

Svane *et al.* reported on their phase I immunization study in breast cancer patients with p53 peptide pulsed DC [41]. Autologous dendritic cells were pulsed with three wild-type and three modified HLA-A2 restricted p53 peptides combined with a MHC class II binding peptide (PADRE). Patients received ten subcutaneous immunizations with at least 5 x 10^6 peptide pulsed dendritic cells combined with 6 mIU/m^2 interleukin 2 (IL-2). Two out of six patients had a clinical response and three out of six developed p53 specific T-cell responses (including the two patients with a clinical response), without significant toxicity.

The phase II study performed by Svane *et al.* [42] was carried out in direct continuation of their phase I study using the same vaccination regime as described above. Only five out of 26 patients completed all ten planned immunizations due to rapid progression of disease or death. Positive immunohistochemical staining of p53 by the primary tumor was found more frequently in patients achieving stable disease during treatment, indicating an effect of p53-specific immune

therapy. However, immunohistochemical staining for p53 might underestimate the patients' ability to present p53 at its tumor cell surface, as tumors in which p53 is inactivated indirectly through binding to viral proteins for example, will not score positive for p53, but can be recognized by CTLs [1;2]. In most cases, an increase in the number of p53-specific CTLs during vaccination was measured, however a tendency towards a more marked decline at late time points after vaccination was observed. However, these heavily pre-treated metastatic breast cancer patients with a high tumor burden are not the ideal patient group to translate p53 specific activation of the immune system into significant tumor regression.

Dendritic cell-based vaccines are laborious in production and restricted to individual patients, but have the advantage that DCs are highly efficient APCs [43]. A significant fraction of the advanced stage breast cancer patients obtained disease stabilization and induction of p53-specific immunity during p53-DC vaccination. Type and maturation status of DCs are issues to be solved in future studies with this vaccination approach. Moreover, further clinical studies should be performed at an earlier stage of disease with progression free survival or overall survival as an endpoint.

PEPTIDE-BASED VACCINES

SHORT PEPTIDES

Since the first identification of a defined tumor-specific CTL epitope, the concept of immunizing cancer patients with a single synthetic peptide epitope has been elaborated (reviewed in [44-46]). The relatively poor immunogenicity of peptide epitopes requires them to be injected together with adjuvants. Important advantages of short peptide vaccination are its defined nature and easy manner to synthesize.

Lomas et *al.* performed a phase I trial targeting several p53-overexpressing solid cancer types in 14 patients with an idiotypic vaccine, composed of a pool of eight peptides derived from the complimentarity determining regions (CDRs) of human anti-p53 antibodies admixed with granulocyte-macrophage colony-stimulating factor (GM-CSF) [47]. None of the trial patients was found to have vaccine-specific, IFN-γ-secreting T-cells as assessed by ELISPOT assay. However, a vaccine induced response was observed in 2 out of 6 patients in the proliferation assay. Clinical responses were not registered and only CTC I/II toxicities were observed.

Rahma et *al.* compared subcutaneous wild-type p53 epitope (264-272) vaccination with intravenous peptide-pulsed DC administration in 21 ovarian cancer patients combined with IL-2 adjuvant in a randomised phase II study. IL-2 administration resulted in directly induced expansion of Tregs and in grade II/IV adverse events in both arms of the study, which was thereafter omitted from the regimen for these patients [48-50]. P53-specific T-cells were observed in approximately 70% of patients, irrespective of whether short peptides or peptide-pulsed DCs were used.

Recent insights in short peptide vaccination have indicated that vaccination with short exact MHC class I binding peptides dissolved in chemical adjuvants, in contrast to peptide-pulsed DCs, is suboptimal mainly because short peptides load exogenously onto MHC class I molecules, including those of non-professional antigen-presenting cells [51].

LONG PEPTIDES

Another vaccination strategy is the use of long peptides encoding the whole p53 protein. The advantage of using long peptides is that, if delivered in the appropriate adjuvant (with APC stimulatory capacity), all potential MHC class I and MHC class II epitopes within the delivered peptides will be processed and presented to host T-cells. These long peptide vaccines are independent of MHC binding motif prediction or processing algorithms and can be administered to subjects independent of their MHC type (reviewed in [51]).

A phase I/II trial using wild type p53 derived synthetic long peptides (SLP) in ovarian cancer was performed by Leffers *et al*. [52]. Twenty patients with recurrent elevation of CA-125 were included and immunized with 10 overlapping p53-SLP in Montanide ISA51. IFN-γ producing p53-specific T-cell responses were induced in all patients who completed the vaccination-scheme as measured by IFN-γ ELISPOT. Vaccine-induced p53-specific T-cells are mediated predominantly by Th2-cells as determined by cytokine bead array, and are capable of migration into immunization sites. The number of Tregs remained constant before and after immunization. Stable disease was observed in 2 out of 20 patients, although no relationship was determined with vaccine-induced immunity.

Speetjens *et al.* used the same p53-SLP vaccine (Leffers *et al*.) in a phase I/II trial, vaccinating ten metastatic colorectal cancer patients [53]. P53-specific T-cells isolated from the vaccination site were characterised as Th-cells which displayed a mixed T-helper 1 and 2 cytokine profile with varying percentages of IFN- and IL-2 producing p53 specific T-cells as determined by cytokine bead array. No overt induction of p53-specific Tregs after p53-vaccination was found. Furthermore, in 6 out of 9 patients, strong proliferative p53-specific T-cell responses were observed in blood samples taken ~ 6 months after the last vaccination.

Peptide based-vaccines have the advantage that antigen-specific immune responses can be easily monitored as a tool to improve the vaccine or vaccination strategy [46]. However, vaccination with short peptides is far from optimal because it can lead to immunological tolerance of the immunizing antigens because T and B cells, in contrast to properly activated DC, lack the co-stimulatory surface molecules required for appropriate effector CTL generation [54-58]. In addition, immunizations with short-peptide vaccines may induce outgrowth of antigen loss variants of the tumor [59]. Furthermore, a single peptide epitope induces either Th-cells or CTL and responses to such epitopes are limited to patients with specific MHC types capable of presenting the peptide used [51]. Limited humoral, cellular and clinical responses were shown in patients immunized by short-peptide vaccines.

In contrast IFN-γ producing p53-specific T-cell responses were induced in the majority of patients receiving long-peptide vaccination. This is probably attributable to the fact that the T-cell epitopes in the long peptide vaccine are efficiently processed and presented by dendritic cells and that the response induced by this vaccine is not restricted to one MHC type. Despite the induction of p53-specific T-cell immunity in vaccinated patients, the p53 long peptide vaccines so far have not induced clinical efficacy. Long peptide vaccines targeting p53 therefore, should be combined with other forms of treatment to eliminate potential mechanisms of immune failure.

3. PERSPECTIVES

Thus far, p53-targeting therapeutic vaccination strategies in cancer patients including administration of recombinant viral vectors, peptide pulsed dendritic cells, short peptide and long peptide vaccines have not shown consistent and/or convincing clinical efficacy.
Whereas some of these vaccines, in particular viral vectors and short peptides have intrinsic shortcomings, a likely explanation for the lack of efficacy is that, despite induction of p53-specific $CD4^+$ T-helper (Th) cells and the recruitment of $CD8^+$ cytotoxic T-lymphocytes (CTLs) to the tumor, a robust anti-tumor response is not accomplished due to immunoregulatory mechanisms counteracting effective T cell-mediated tumor cell killing. T cells that effectively home to tumor metastases can be dysfunctional, pointing toward immunosuppressive mechanisms in the tumor microenvironment [60]. T cell anergy due to insufficient B7 co-stimulation, extrinsic suppression by regulatory myeloid and regulatory T cell populations, inhibition by ligands such as programmed death ligand-1, metabolic dysregulation by enzymes such as indoleamine-2,3-dioxygenase, and the action of inhibitory factors such as TGF- have all been implicated in the lack of efficacy [37;61].
Because of the disappointing clinical results induced by the p53-vaccines, we can conclude that the immunogenicity of these vaccines needs to be enhanced by improving the robustness of the induced effector T cell responses and by effectively disrupting the counterproductive immunoregulation [62]. It may also be useful to simultaneously target additional tumor antigens [63]. Below we discuss several straightforward clinically applicable methods that have been proposed to augment immunogenicity and clinical efficacy of immunotherapeutic vaccines.

ELIMINATING REGULATORY T-CELLS BY CYCLOPHOSPHAMIDE

As mentioned above, the observed lack of clinical efficacy may be partly attributed to the presence of $CD4^+$ $FoxP3^+$ regulatory T-cells (Tregs). It is becoming apparent that immunotherapy itself can induce and/or boost Tregs and that these vaccine-induced Tregs are associated with treatment failure [64-68]. Immunosuppression mediated by Tregs is a major hurdle for successful tumor immunotherapy as Tregs suppress antigen specific T cell responses [60;65-67]. Strategies to eliminate or suppress Tregs to improve clinical efficacy of immunotherapy vary from treatment with commonly used chemotherapeutic agents, such as cyclophosphamide, fludarabine or COX-2 inhibitors, next to direct targeting of Tregs by monoclonal antibodies [69-76].
Low-dose cyclophosphamide is easy to incorporate into a clinical setting. Dosages of cyclophosphamide used in combination with immunotherapy are generally insufficient for cytotoxic reductions of tumor burden, but reduce numbers of Tregs and impair their functionality without deleting other immune cells [69;77-79]. Furthermore, a cohort study in metastatic pancreatic cancer showed an enhanced induction of antigen-specific T-cells in patients pre-treated with cyclophosphamide compared to patients who were not pre-treated with cyclophosphamide. Additionally, median overall survival of patients treated with cyclophosphamide was almost twice as high as that of patients who did not receive cyclophosphamide. This was similar to results obtained with second-

line therapy for metastatic pancreatic cancer [80]. Although the number of circulating Tregs in the patient group vaccinated with the p53-SLP by Leffers et *al.*, is relatively low (7.0%), their presence and recruitment to the tumor may nevertheless foster tolerance to the tumor. We have started a new clinical trial in which p53-SLP immunization is combined with low-dose cyclophosphamide to test whether this increases immunity and clinical activity.

IMMUNOPOTENTIATION BY ANTI-CTLA-4

Another immunopotentiation strategy that has been used in the clinical setting is blockade of cytotoxic T-lymphocyte antigen-4 (CTLA-4) aiming to counteract inhibitory signals in order to induce antitumor immunity. CTLA-4 is a co-stimulatory molecule expressed on activated T-cells that delivers an inhibitory signal which reverses the T-cell response, resulting in anergy [81]. Two human anti-CTLA-4 monoclonal antibodies (mAbs), MDX-010 (ipilimumab) and CP-675,206 (tremelimumab), have thus far been used in clinical trials with encouraging results in patients with melanoma, lymphoma, and urothelial carcinoma of the bladder [74;82-85]. Anti-CTLA-4 mAbs are well poised to be combined with other therapies. Moreover, these antibodies may enhance the effectiveness of other therapies like cancer vaccines when used in combination. Therefore, several clinical trials on antitumor regimens added anti-CTLA-4 to their treatment regime, aiming to improve clinical efficacy in the participating patients [86-88].

Anti-CTLA-4 mAbs have shown antitumor activity, however accumulating evidence indicates that anti-CTLA-4 mAbs paradoxically increases the number of Tregs, thereby hampering the effect of anti-CTLA-4 [89]. This has stimulated interest in designing clinical trials using anti-CTLA-4 mAbs in combination with Treg controlling strategies to improve clinical outcome. Several promising preclinical studies combining anti-CTLA-4 with Treg depletion have been conducted so far [90-92]. The studies of combined immunopotentiating low-dose cyclophosphamide and anti-CTLA-4 provides the foundation for integrating immunotherapy with other targeted therapies for the treatment of patients with advanced stage cancer.

IMMUNOSTIMULATION BY CHEMOTHERAPEUTIC REGIMENS

The interaction of tumor cell death due to chemotherapy on one hand and induction of anti-tumor immune responses induced by this cell death on the other hand might be essential to achieve the optimal result in tumor eradication [93-95]. It is postulated by Zitvogel et al that activation of the calreticulin exposure pathway is an important mechanism of activation of the immune system after treatment with classical therapies like chemotherapy [96]. They thought that chemotherapy in general results in a strong reduction of major components of the immune system and thereby harming the immune system ready to attack the tumor does not hold true anymore. Evolving evidence shows the opposite. Immunotherapy in combination with chemotherapy might be a very effective strategy as induction of long lived antigen specific memory T cells recently have been identified [97]. Cisplatinum next to paclitaxel and doxorubicin, drugs often used in gynaecologic malignan-

cies, reportedly make tumor cells more susceptible to Granzyme B dependent killing by cytotoxic T cells [35]. It is attractive to use these immunomodulatory effects of chemotherapy by combining it with p53-specific immunization.

NEW VACCINATION STRATEGY: MULTI-EPITOPE VACCINES

Most clinical studies included in this review targeted only p53, limiting the use of such vaccines to those patients with (over)expression of this specific tumor antigen. Furthermore, tumor cells might lose antigens and therefore display a reduced susceptibility to vaccine-induced immunity in the course of the vaccinations. Immunization using a cocktail of antigens has been proposed as a "universal" vaccine strategy [98]. As solid tumors often show heterogeneous protein expression, multi-antigen vaccines may have greater therapeutic potential which can compensate for tumor antigen-loss variants [63;99]. The ability to target multiple antigens may also improve the immunogenicity of therapeutic vaccines. We believe that addition of other tumor antigens to the p53-vaccine might ultimately result in an enhanced clinical effect [98]. Particularly because the CTL repertoire against p53 based on mouse studies and observations in patients, appears to be more deeply tolerized than the Th cell repertoire [24]. Addition of immunotherapy against antigens that more readily elicit tumoricidal CTL responses may therefore fully exploit the excellent ability of p53 vaccination to elicit Th cell responses.

Thus far, several clinical trials targeting multi-antigens have been conducted. Kirkwood *et al.* reported that the effect of a multi-epitope melanoma vaccine tested in a phase II trial is correlated with prolonged survival in metastatic melanoma patients. Addition of immunomodulatory cytokines had no beneficial effect on prognosis [100]. A multi-antigen vaccine tested in prostate cancer patients resulted in long-term stable disease [101].

Recently, a p53 comprising multi-epitope vaccine has been administered to malignant melanoma patients in a phase I/II clinical trial. Results of the DC-vaccine pulsed with p53, survivin and telomerase-derived peptides in combination with low-dose IL-2, have been published by Trepkiakas *et al.* [102]. This group previously targeted p53 in a DC vaccination trial as described in this review [41;42]. Due to this new multi-antigen pulsed DC-vaccine, stable disease correlated with prolonged survival suggesting a clinical benefit. Nevertheless, significant changes in Treg frequencies during treatment were seen and ascribed to IL-2 administration. Consequently, IL-2 was removed from their DC vaccination strategy and replaced by low-dose cyclophosphamide in an ongoing clinical trial in melanoma patients in order to enhance the immune and clinical responses.

Addition of multiple antigens in an immunotherapeutic vaccine will enhance the barrier against escape of antigen loss variants of the tumor and will exploit more fully the antitumor CTL potential of the patient. Future studies on multi-epitope immunotherapy moreover applicable in a higher percentage of patients, is expected to result in a significantly enhanced efficacy of anticancer immunotherapy.

4. CONCLUSION

Over the past decade, several studies on p53-vaccines for immunotherapeutic treatment of cancer patients have been conducted. Different vaccination strategies varying from viral vectors, dendritic cells, short and long peptides have been used. Of these vaccination modalities, viral vectors and short peptides suffer from major drawbacks. Although peptide-loaded DC and long peptides have induced reasonably strong p53-specific immune responses, in particular $CD4^+$ T cell responses, robust clinical responses so far have failed to materialize. In this review we point out that the limited clinical efficacy dictates further exploration of new immunization strategies. P53-vaccines can easily be combined with low-dose cyclophosphamide, anti-CTLA-4, chemotherapeutic regimens or other tumor antigens, as immunopotentiation treatment modalities. An integrative immunotherapeutic strategy combining 'up-front' Treg cell ablation followed by p53 vaccination may limit generation of new tumor-sensitized Tregs and therefore, might improve the clinical responses in cancer patients. Moreover, addition of multiple antigens to the p53-vaccine will make it applicable in a higher percentage of patients and will exploit the anticancer T-cell response. Future studies will be needed to establish the best combination of therapy and to identify cancer patients most likely to respond to combined anti-p53 therapies.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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CHAPTER 5

POTENTIAL TARGET ANTIGENS FOR A UNIVERSAL VACCINE IN EPITHELIAL OVARIAN CANCER

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ABSTRACT

The prognosis of epithelial ovarian cancer (EOC), the primary cause of death from gynaecological malignancies, has only modestly improved over the last decades. Immunotherapeutic treatment using a cocktail of antigens has been proposed as "universal" vaccine strategy. We determined the expression of tumor antigens in the context of MHC class I expression in 270 primary tumor samples using tissue microarray. Expression of tumor antigens p53, SP17, survivin, WT1 and NY-ESO-1 was observed in 120 (48.0%), 173 (68.9%), 208 (90.0%), 129 (56.3%) and 27 (11.0%) of 270 tumor specimens, respectively. In 93.2% of EOC at least one of the investigated tumor antigens was (over)expressed. Expression of MHC class I was observed in 78.1% of EOC. In 3 out 4 primary tumors (over)expression of a tumor antigen combined with MHC class I was observed. These results indicate that a multi-epitope vaccine, comprising these antigens, could serve as a universal therapeutic vaccine for the vast majority of ovarian cancer patients.

INTRODUCTION

Epithelial ovarian cancer (EOC) is the most common cause of death in gynaecologic malignancies [1]. Most ovarian cancer patients are asymptomatic until disease has metastasized and therefore two-third of all patients are diagnosed with advanced stage disease [1;2]. Although the majority of patients with advanced disease achieve complete clinical response rates due to the current therapy of aggressive cytoreductive surgery and platinum-taxane based chemotherapy, more than 90% develop tumor recurrence, resulting in five-year survival rates of only 30% [3].

These records express the need for a new and improved therapy for EOC. The significance of the immune response for the clinical course of EOC has led to attempts to modulate it artificially with (antigen specific) immunotherapeutic strategies [4]. Presentation of tumor antigens in the context of MHC molecules on tumor cells is critical for the efficacy of targeted immunotherapy [5]. Thus far, approaches at therapeutic vaccination in cancer patients including administration of peptide pulsed dendritic cells, recombinant viral vectors encoding tumor antigen, DNA-fusion vaccine and single peptide vaccine have not shown consistent and high percentages of clinical successes [6-12]. Most clinical studies on immunotherapy targeted one antigen, limiting the use of such vaccines to those patients with (over)expression of that specific tumor antigen. Immunization using a cocktail of antigens has been proposed as a "universal" vaccine strategy. Whereas solid tumors often show heterogeneous protein expression, multi-antigen vaccines may have greater therapeutic potential and compensate for tumor antigen-loss variants [13;14]. The ability to target multiple antigens, may also improve the immunogenicity of therapeutic vaccines [13;15]. Therefore discovery of multiple tumor antigens in EOC may provide opportunities for multi-antigen immunotherapeutic strategies that can induce sufficient clinical responses. Tumor antigens that are inherently immunogenic and oncogenic in ovarian cancer are p53 [16-18], Sperm Protein 17 (SP17) [14;19;20], Wilms' tumor gene (WT1) [21-23], Survivin [24-26] and NY-ESO-1 [12;27;28].

The presence of a α (heavy) chain and $ß_2$-microglobulin is a prerequisite for the formation of a stable MHC class I complex [29]. Such stable MHC class I complexes are required for presentation of the tumor antigenic peptides [30].

No reports have been published describing tissue microarray staining of p53, SP17, survivin, WT1 and NY-ESO-1 with MHC class I expression in EOC. Further knowledge of the expression of multiple tumor antigens in the context of MHC class I expression is necessary to develop strategies to increase clinical efficacy of multi-antigen immunotherapy in EOC.

Aim of the present study was to investigate the expression of SP17 and NY-ESO-1 and overexpression of p53, WT1 and survivin together with $ß_2$-microglobulin and the α-chains, HLA-A and HLA-B/C in tumor samples obtained from a large, well-documented cohort of primary EOC patients using tissue microarray.

MATERIALS AND METHODS

PATIENTS

Since 1985 the Department of Gynecological Oncology of the University Medical Centre Groningen (UMCG) prospectively stores all clinicopathologic and follow-up data of epithelial ovarian cancer patients in a computerized database. Tumor samples from 361 patients were collected on a tissue microarray. This tissue microarray contains primary ovarian tumor tissue obtained before chemotherapy of 270 patients. Patients with borderline or non-epithelial tumors were excluded. Primary treatment for all patients consisted of surgery and adjuvant chemotherapeutic treatment consistent of platinum-based regimens and others. Since 1995 platinum-based chemotherapy was supplemented by taxanes.

In the current study the 270 EOC patients were selected for tumor antigen analysis who underwent primary surgery between 1985 and 2006 and of whom sufficient paraffin-embedded ovarian tumor tissue and complete follow-up data were available. In a non-selected subgroup of 183 primary EOC patients MHC class I expression was analyzed. These data are partly previously published by our group [29].

Patients were surgically staged according to FIGO (International Federation of Gynecology and Obstetrics) classification [31]. Optimal and suboptimal debulking was defined as the largest residual tumor lesions having a diameter of respectively <2 cm or ≥2 cm. Histology of all tumors was determined according to World Health Organization criteria [32].

All relevant data were filed in a separate anonymous database in which patients were given unique codes to protect patient identity. Database management was restricted to two people with access to the larger database containing all patients' characteristics. Due to these procedures no additional patient or institutional review board approval was required according to Dutch Law.

TISSUE MICROARRAYS

Tissue microarrays were constructed as described previously [17]. Four cores of 0.6mm² were taken by biopsy and placed by a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA) on a recipient paraffin block. Using a microtome, 4 µm sections were cut from each tissue microarray block and applied to aminopropyltriethoxysilane-coated slides. All arrayed samples were H&E-stained to confirm the presence of tumor tissue.

IMMUNOHISTOCHEMICAL STAINING OF TISSUE MICROARRAYS

Tissue microarray sections were de-paraffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water. The sections were boiled for 15 minutes in a microwave to accomplish antigen retrieval. Endogenous peroxidase was blocked by incubation of sections for 30 minutes in 0.3% hydrogen peroxide. Primary antibodies, antigen retrieval buffers and detection methods used, are provided as supplementary data (Table 1). Sections were counterstained with hematoxylin. All control experiments gave satisfactory results.

SCORING

Evaluation of immunostaining was independently performed by two observers blinded to the clinical data. Agreement between the two observers was >90%. Contradictory outcomes were reviewed by a gynecological pathologist and were reassigned by approval of all parties.
Immunostaining for p53, HLA-A, HLA-B/C and $ß_2$-m was scored as described in previous studies [17;29;30]. The immunohistochemical reaction for SP17 [33], WT1 [34;35], survivin [24;36;37] and NY-ESO-1 [38] was semi-quantitatively graded into four classes based on the frequency of nuclear staining for SP17, WT1 and survivin, and cytoplasmatic staining in NY-ESO-1 in ovarian cancer cells: negative = no/ very low frequency (<5%) immunopositive cells; + = low frequency (≤ 5-25%); ++ = moderate frequency (25%-50%); +++ = high frequency (50%-75%); ++++ = very high frequency (75-100%). The cutoff was 'a priori' chosen for scoring: cases with low frequency or higher were considered positive for tumor antigen expression.

STATISTICAL ANALYSIS OF DATA

Statistical analysis was carried out using the SPSS 16.0 software package for Windows (SPSS Inc., Chicago, USA). All cases with <2 evaluable cores were excluded from analysis.

Table 1 **Antibodies used for immunohistochemical staining**

Antigen	Antigen retrieval	Clone	Dilution	Company
p53	Tris/EDTA (pH8)	DO-7[1]	1:2000	DAKO[2]
SP17	Citrate (pH 6)	Sp17MF1	1:100	[3]
survivin	Citrate (pH 6)	71G4B7E	1:100	Cell signaling[4]
WT1	Tris/HCL (pH9)	6F-H2	1:25	DAKO[2]
NY-ESO-1	EDTA (pH 8)	E978	1:50	Zymed[5]
HLA-A	Citrate (pH 6)	HCA2	1:500	[6]
HLA-B/C	Citrate (pH 6)	HC-10	1:100	[6]
$ß_2$-m	Citrate (pH 6)	Polyclonal	1:400	DAKO[2]

[1] Detects both wild-type and mutant p53 protein; [2] DAKO, Glostrup, Denmark; [3] The SP17 antibody kindly provided by Dr. Maurizio Chiriva, Texas Tech University; [4] Cell Signaling, Danvers, USA; [5] Zymed, San Francisco, USA; [6] The HCA2 and HC-10 antibodies were a gift from Prof. Dr. Neefjes, Netherlands Cancer Institute, Amsterdam, The Netherlands.

RESULTS

PATIENTS

Tumor samples from 270 consecutive primary ovarian cancer patients (median age 56.9 years, range 16-89) treated at the UMCG between 1985 and 2006 were available (Table 2). The majority of patients presented with serous histology, advanced stage, and/or high grade disease. First-line chemotherapy regimens were platinum-based in 90 (34.2%) patients and platinum- and taxane-based in 108 (41.1%) patients. Other regimens were given to 25 (9.5%) patients, while 40 (15.2%) patients did not receive chemotherapy because of early stage disease, co-morbidity or treatment refusal.

TUMOR ANTIGEN (OVER)EXPRESSION IN EOC

P53, SP17, survivin, WT1 and NY-ESO-1 (over)expression was observed in 48.0%, 68.9%, 90.0%, 56.3% and 11.0% of tumors, respectively (Table 3). In 93.2% tumors at least one of the investigated tumor antigens was (over)expressed (Table 4). Expression of only one tumor antigen was found in 40 (15.2%) tumors, 70 (26.6%) tumors expressed two antigens, 70 (26.6%) tumors expressed three antigens, 58 (22.1%) tumors expressed four antigens and 7 (2.7%) tumors expressed all five investigated tumor antigens. Absence of expression of any antigen was seen in 18 (6.8%) patients. Non-evaluable primary tumors due to core loss during staining procedures or absence of tumor tissue ranged from 19 (7.4%) for SP17 staining to 41 (15.2%) for WT1 staining. Several specific combinations of tumor antigen expression cover high percentages of EOC patients, varying from 95.5% (214/224) combining two antigens to a maximum coverage of 98.2% (216/220) combining four antigens (Table 5).

IMMUNOSTAINING MHC CLASS I

Co-expression of HLA-A and $ß_2$-m or HLA-B/C and $ß_2$-m was observed in 98 (53.6%), and 136 (74.7%) of the tumors, respectively (Table 3). Positive MHC class I expression, defined as HLA-A and $ß_2$-m and/or HLA-B/C and $ß_2$-m co-expression, was observed in 143 (78.1%) tumors.

CO-EXPRESSION OF TUMORANTIGENS AND MHC CLASS I IN EOC

Of all EOC positive for p53, SP17, survivin, WT1 or NY-ESO-1, 82.5%, 82.8%, 77.0%, 80.9% and 80.0%, were also positive for MHC class I, respectively (Table 6). In 78.4% of tumors (over)expressing one or more tumor antigens, also expression of MHC class I was found. Furthermore, 74.3% of all tumors co-expressed MHC class I and at least one tumor antigen.

Table 2 Patient and tumor characteristics

	All patients (n = 270)
Age (years)	
Mean (SD)	56.9 (13.8)
	n (%)
FIGO stage*	
Stage I	67 (24.9)
Stage II	26 (9.7)
Stage III	144 (53.5)
Stage IV	32 (11.9)
Missing	1
Tumor type	
Serous	147 (59.8)
Mucinous	37 (15.0)
Endometrioid	42 (17.1)
Clear cell	17 (6.9)
Undifferentiated	3 (1.2)
Missing	24
Differentiation grade	
Grade I	51 (20.2)
Grade II	77 (30.6)
Grade III	113 (44.8)
Undifferentiated	11 (4.4)
Missing	18
Residual disease	
<2 cm	155 (59.0)
≥2 cm	94 (35.7)
Positive**	21 (5.3)

*FIGO = International Federation of Gynecology and Obstetrics.
** Amount unknown

Table 3 Expression levels of antigen and MHC class I components

	P53[1]	SP17[1]	Survivin[1]	WT1[1]	NY-ESO-1[1]
	n (%)	n (%)	n (%)	n (%)	n (%)
Normal/negative	130 (52.0)	78 (31.1)	23 (10.0)	100 (43.7)	219 (89.0)
Overexpression/positive	120 (48.0)	173 (68.9)	208 (90.0)	129 (56.3)	27 (11.0)
Missing	20	19	39	41	24
	HLA-A$^+$ /β_2-m^{+2}	HLA-B/C$^+$ / β_2-m^{+2}	MHC class I[3]		
Positive[4]	98 (53.6)	136 (74.7)	143 (78.1)		
Negative[4]	85 (46.4)	46 (25.3)	40 (21.9)		
Missing		1			

[1] primary EOC patients, $n = 270$; [2] staining in subgroup, $n = 183$; [3] MHC class I expression is defined as HLA-A and β_2-m and/or HLA-B/C and β_2-m co-expression; [4] positive is both components HLA-A/B/C and β_2-m expressed, negative are all other phenotypes

Table 4 Expression of single or multiple antigens in EOC

Number of antigens	*n*	%	Cumulative %
1	40	15.2	15.2
2	70	26.6	41.8
3	70	26.6	68.4
4	58	22.1	90.5
5	7	2.7	93.2
none	18	6.8	100.0
missing	7		

$n = 270$

Table 5 Expression of specific antigen combinations in EOC

Antigen combinations	% (n/total)
One antigen	
p53	48.0 (120/250)
SP17	68.9 (173/251)
survivin	90.0 (208/231)
WT1	56.3 (129/229)
NY-ESO-1	11.0 (27/246)
Two antigens	
p53, SP17	84.2 (203/241)
p53, survivin	95.5 (214/224)
p53, WT1	73.1 (163/223)
p53, NY-ESO-1	52.7 (125/237)
SP17, survivin	94.7 (215/227)
SP17, WT1	82.3 (186/226)
SP17, NY-ESO-1	74.0 (179/242)
survivin, WT1	93.0 (212/228)
survivin, NY-ESO-1	90.8 (208/229)
WT1, NY-ESO-1	60.3 (138/229)
Three antigens	
p53, SP17, survivin	97.7 (217/222)
p53, SP17, WT1	91.4 (202/221)
p53, SP17, NY-ESO-1	86.4 (203/235)
p53, survivin, WT1	95.9 (213/222)
p53, survivin, NY-ESO-1	95.5 (213/223)
p53, WT1, NY-ESO-1	74.4 (166/223)
SP17, survivin, WT1	96.0 (216/225)
SP17, survivin, NY-ESO-1	95.6 (216/226)
SP17, WT1, NY-ESO-1	84.5 (191/226)
survivin, WT1, NY-ESO-1	93.4 (213/228)
Four antigens	
p53, SP17, survivin, WT1	98.2 (216/220)
p53, SP17, survivin, NY-ESO-1	97.7 (216/221)
p53, SP17, WT1, NY-ESO-1	92.3 (204/221)
p53, survivin, WT1, NY-ESO-1	95.9 (213/222)
SP17, survivin, WT1, NY-ESO-1	96.4 (217/225)
All antigens	
p53, SP17, survivin, WT1, NY-ESO-1	98.2 (216/220)

n = 270

Table 6 Co-expression of MHC class I components with tumorantigens

	p53⁺ *n* = 80	**SP17⁺** *n* = 116	**Survivin⁺** *n* = 152	**WT1⁺** *n* = 89	**NY-ESO1⁺** *n* = 20	**Tumorantigen⁺²** *n* = 173
	n/total *(%)*	n/total *(%)*	n/total *(%)*	n/total *(%)*	n/total *(%)*	n/total *(%)*
HLA-A⁺ ß$_2$m⁺ *n* = 98	52/80 *(65.0)*	64/116 *(55.2)*	80/152 *(52.6)*	50/89 *(56.2)*	13/20 *(65.0)*	96/173 *(55.0)*
HLA-B/C⁺ ß$_2$m⁺ *n* = 136	62/78 *(79.5)*	94/116 *(81.0)*	111/151 *(73.5)*	67/89 *(75.3)*	15/20 *(75.0)*	129/171 *(75.4)*
MHC class I⁺ *n* = 143	66/80 *(82.5)*	96/116 *(82.8)*	117/152 *(77.0)*	72/89 *(80.9)*	16/20 *(80.0)*	136/173 *(78.4)*
All patients[1] *n* = 183	66/174 *(37.9)*	96/178 *(53.9)*	117/169 *(69.2)*	72/167 *(43.1)*	16/175 *(9.1)*	136/183 *(74.3)*

[1] Antigen⁺ and MHC class I⁺ in all subgroup patients (n = 183); [2] ≥ 1 tumorantigen expression

DISCUSSION

In a large, well-documented cohort of representative EOC patients (over)expression of at least one of the tumor antigens p53, SP17, survivin, WT1 or NY-ESO-1 was observed in over 90% of the tumors. To our knowledge this is the first study on the expression of multiple tumor antigens in a large cohort of EOC. Only a minority (6.8%) of the tumors did not express one of the selected tumor antigens. About 75% of the EOC tumors expressed both, one of the tumor antigens and MHC class I. This observation underlines the relevance of designing a multi-epitope vaccine consisting of p53, SP17, NY-ESO-1, survivin and WT1 for the immunotherapeutic treatment of ovarian cancer.

This inventory tissue microarray study enables us to analyze the expression of five well known tumor antigens in EOC, in correlation to MHC class I expression. Tissue microarray is a practical and powerful tool for high-throughput analysis of tumor tissue identifying targets in human cancers [39]. P53, SP17, NY-ESO-1, survivin and WT1 are immunogenic target antigens in EOC. Rates of observed (over)expression of p53, SP17, survivin and WT1 in 48.0%, 68.9%, 90.0% and 56.3% of EOC patients, respectively, are in agreement with previous studies [16;24;40;41]. NY-ESO-1 expression was seen in 11.0% of tumors in our cohort which is in agreement with the results of others [42;43]. However, Odunsi *et al* observed NY-ESO-1 expression in 43% of EOC patients [38;44]. This difference in expression might be explained by considerable methodological variability among the different studies. The type of study design, antibodies and assays used to study NY-ESO-1 expression, determination of cut-off points for aberrant NY-ESO-1 expression and the definition of study end points vary greatly among different studies. Immunohistochemical analyses of tumors have shown heterogeneous NY-ESO-1 expression [45]. Since expression of NY-ESO-1 is mostly focal and non-uniform, tissue microarrays containing large numbers of tumor tissue are essential to determine NY-ESO-1 expression in EOC. Our sample size of 270 EOC patients might be more potent to distinguish between positive and negative NY-ESO-1 expression in EOC compared to 143 EOC patients analyzed by Odunsi *et al*.
We previously reported on the expression of tumor antigens EGFR and Her-2 in our large, well-documented cohort of representative EOC, using tissue microarray [46]. EGFR and Her-2 overexpression was observed in 7.0% and 5.2% of EOC, respectively. The expression of EGFR and Her-2 has been extensively studied in ovarian cancer [47;48]. Aberrant activity of these antigens is important in tumor growth and development [49;50]. Therefore, EGFR and Her-2 were considered to be attractive targets for immunotherapeutic strategies in EOC. Because of the low expression levels in EOC, therapeutic potential of vaccines targeting EGFR and Her-2 is limited. As the existing repertoire of known antigens in EOC is relatively small, we performed our innovative study on five highly expressed tumor antigens which may provide opportunities for multi-epitope immunotherapeutic strategies targeting the majority of EOC patients.

We provide first evidence that several antigen combinations can be used in a multi-epitope vaccine for EOC treatment, since different antigen combinations cover high percentages of EOC patients. Vaccines comprising a mixture of for example p53, SP17 and survivin or combining survivin, WT1

and NY-ESO-1 cover the vast majority of EOC patients. Maximum coverage of EOC patients can be obtained by a vaccine comprising four antigens p53, SP17, survivin and WT1.
Single antigen vaccines targeting p53 [51], SP17 [40], NY-ESO-1 [52], survivin [11] and WT1 [22] have been described to generate tumor antigen-specific cytotoxic T-cell lymphocytes (CTLs) able to lyse autologous tumor cells. One can envision that multi-epitope vaccines may enhance immunogenicity, improving clinical efficacy of the immunotherapeutic vaccine.
Multi-epitope vaccines should preferably contain multiple MHC class I-presented CTL epitopes derived from different target antigens together with a tumor-specific MHC class II-presented T-helper epitope. This will reduce the risk of immune-driven selection of antigen-loss variants of the tumor. Next, given the pivotal role of T-helper cells in promoting the primary and secondary CTL responses through the induction of DC maturation and the production of cytokines, the inclusion of T-helper epitopes in a multi-epitope based vaccine will have strong beneficial effects [6]. For example, p53-specific T-helper cells induced upon p53 specific immunization might fulfil this role [53].
Important advantages of well-defined multi-epitope vaccines over non-defined vaccines, such as tumor lysate vaccines, are their defined nature [6;54], lack of suppressive inducing antigens [55-57], simple way of manipulation to prevent dominance of one antigen over the others [6;58], universal applicability [6;59], easiness to make in a standardized procedure [59;60], possibility to combine with other strategies [59] and limited autoimmune toxicity [55;61].
Moreover, administration of a multi-epitope vaccine as a single mixture offers advantages including: (1) injection of a limited volume, (2) lower the number of skin sites with local toxicity due to injection site reactions, and (3) lower chance of error and contamination with the preparation of one versus multiple epitope preparations [13;14].
In contrast, previous studies showed that administration of multiple epitopes at one injection site could lead to a more vigorous response to just one of the involved antigens [62;63]. We reasoned that this disadvantageous result might be due to immunodominance of one antigen over the other. Preclinical studies might be helpful in designing the optimal combination of multi-antigen vaccines, trying to predict and / or prevent immunodominance. In contrast, the synergy between antigens included in a multi-epitope vaccine might induce immune responses with increased potency compared with the response induced by the same epitopes individually [6]. Separate injection sites for all of the involved antigens may result in a significant increase in the magnitude of the antigen specific T-cell response. It still holds true that several multi-antigen combinations cover high percentages of tumors. The most favourable vaccine, based on (pre)clinical studies concerning immunodominance, can be used for treatment of EOC patients.

MHC class I down-regulation was observed in 21.9% of tumors. Loss of MHC class I molecules on tumor cells, which may lead to immune escape, is often restricted to one or a few alleles. Targeting multiple epitopes restricted by different class I molecules of the patient will likely circumvent such an escape mechanism [6]. The tumor associated antigens p53, NY-ESO-1 and WT1 epitopes are presented both by MHC class I and II (according to listing at www.cancerimmunity.org, update September 2008). As a result p53, WT1 and NY-ESO-1 [64] can function as both CTL and T-helper cell targets.

Considering the importance of the expression of MHC class I by tumor cells for immune recognition by T cells, several regimens could be added in the multi-epitope vaccine to enhance MHC class I expression. Treatment with IFN-gamma is known to upregulate MHC class I [10;65;66]. Another possibility would be addition of demethylating agents to the multi-epitope vaccine, since DNA hyper-methylation, common in human tumors, may result in the loss of MHC class I expression [67;68].
The most promising finding that emerges from this study is that the vast majority of EOC patients present one or more tumor antigens. Furthermore, if tumor cells present one of our investigated tumor antigens it is likely to express MHC class I as well. Therefore, a vaccine comprising the investigated tumor antigens is capable of targeting tumor cells of the vast majority of EOC patients. Since several combinations of tumor antigens cover the majority of EOC patients, different institutes can attribute personally preferred antigens to their multi-epitope vaccine.

In summary, we are first to show that multi-epitope immunotherapy combining tumor antigens p53, SP17, survivin, WT1 and/or NY-ESO-1 might be a promising new therapeutic vaccination strategy in ovarian cancer.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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CHAPTER 6

TUMOR INFILTRATING CYTOTOXIC T-LYMPHOCYTES AS INDEPENDENT PROGNOSTIC FACTOR IN EPITHELIAL OVARIAN CANCER WITH WILMS' TUMOR PROTEIN 1 OVEREXPRESSION

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ABSTRACT

Immune response characterization at the primary tumor site enables the design of therapeutic vaccination strategies with higher efficacy in epithelial ovarian cancer (EOC).

In this study, we related Wilms' tumor protein 1 (WT1) overexpression, a well established immunotherapeutic target, to clinicopathological characteristics, immunological parameters and survival in primary EOC. WT1 overexpression was evaluated in primary EOC tissue of 270 patients by immunohistochemistry on tissue microarrays (TMAs). Clinicopathological characteristics, follow-up and data on infiltration of $CD8^+$ cytotoxic T-lymphocytes (CTLs), $FoxP3^+$ regulatory T-lymphocytes (Tregs), MHC class I, and II molecule expression, were derived from a previously published dataset. WT1 overexpression was defined as positive immunostaining for WT1. WT1 overexpression, present in 56.3% of EOC, was associated with infiltration of Tregs [odds ratio (OR), 2.7; 95% confidence interval (95% CI), 1.6-4.7; $P < 0.001$] and up-regulation of MHC class II (OR, 2.2; 95% CI, 1.2-4.1; $P = 0.014$). Advanced stage (OR, 4.0; 95% CI, 1.9-8.6; $P < 0.001$) and serous histology (OR, 6.7; 95% CI, 3.2-13.6; $P < 0.001$) were independent predictors of WT1 overexpressing EOC. High number of CTL was an independent prognostic factor for progression free survival [hazard ratio (HR), 0.5; 95% CI, 0.3-0.8; $P = 0.006$] in WT1 overexpressing EOC. As WT1-overexpressing EOC is associated with CTL and Treg infiltration next to MHC class II up-regulation future clinical trials should evaluate the combination of therapeutic WT1-vaccines with strategies depleting Tregs and/or up-regulating MHC class I, in an attempt to enhance clinical efficacy in EOC patients.

INTRODUCTION

Due to a lack of specific symptoms most epithelial ovarian cancer (EOC) patients are diagnosed with advanced stage of disease [1;2]. Despite aggressive treatment with cytoreductive surgery and platinum-taxane based chemotherapy tumor recurrences often occur, resulting in a poor overall survival [3;4]. Therefore, new therapeutic strategies are urgently required. Since immune responses positively affect the clinical course of EOC patients, tumor-associated antigen based immunotherapeutic strategies are considered promising innovative treatment modalities in EOC [5]. Wilms' tumor protein 1 (WT1), initially defined as a tumor suppressor gene, is a potential tumor-associated antigen in EOC [6-8]. Studies by other groups previously indicated that WT1 is mainly overexpressed in advanced stage and serous ovarian cancer, and is associated with unfavorable prognosis [8-12], thereby presenting WT1 as a potential target for immunotherapy.

$CD8^+$ cytotoxic T-lymphocytes (CTLs) recognize tumor-associated antigen derived peptides which are presented on the tumor cell surface in association with major histocompatibility complex (MHC) class I molecules, leading to the killing of tumor cells [13]. $CD4^+$ T-helper (Th) cells recognizing tumor-associated antigen derived peptides presented in association with MHC class II molecules on professional antigen presenting cells, play an important role in orchestrating and sustaining the local immune attack by CTL [14;15]. In contrast, inhibitory $CD4^+$ $FoxP3^+$ regulatory T-cells (Tregs) can also recognise MHC class II molecules thereby impeding antitumor immunity by inhibiting CTL activation [16;17]. In case of EOC, we and other groups have shown that high numbers of CTL are indicative of longer survival [18-20]. In contrast Tregs seem to reduce tumor-specific immunity and result in poorer survival of patients with EOC [21;22]. The latter however is not a general finding for EOC and other tumors [23;24]. MHC class I down-regulation have been reported to negatively influence survival in EOC [25-28]. Up-regulation of MHC class II on ovarian cancer cells however, results in contrasting findings considering prognosis [27;28].

Based on promising preclinical results, which strongly suggest the occurrence of WT1-directed immune responses in cancer patients, several clinical trials have been performed targeting WT1 [6;29;30]. Thus far, therapeutic WT1-based vaccination of cancer patients, including EOC patients, have not shown consistent or high percentages of clinical successes [31-36]. These disappointing clinical results may be explained by general features of the cancer patients' immune status, negatively influencing the effectiveness of WT1-immunization. To our knowledge, no reports have been published describing associations of WT1 overexpression with T-lymphocyte infiltration and MHC class I and II expression in a large, well-documented cohort of primary EOC patients. Analyzing immunological parameters at the primary tumor site in the context of antigen expression provides new clues how to modify vaccination strategies to improve immunogenicity and develop clinical efficacy. Aim of the present study therefore was to relate WT1 (over)expression to immunological parameters such as MHC class I and II expression, intratumoral CTL, and Treg infiltration in EOC. We were particularly interested in the presence and prognostic impact of immunological parameters in the WT1 overexpressing subgroup of EOC, as this is the target group of immunotherapeutic WT1 vaccines. We translated our findings into several immunopotentiating combination strategies suitable for clinical use.

MATERIALS AND METHODS

PATIENTS

Since 1985 the Department of Gynecologic Oncology of the University Medical Centre Groningen (UMCG) prospectively stores all clinicopathologic and follow-up data of EOC patients in a computerized database. Samples of ovarian tumor tissue from 361 patients were collected on a TMA among which 270 tumor samples obtained at primary surgery prior to chemotherapy (period 1985-2006), selected for the current study. Data on clinicopathological characteristics, follow-up, survival and immunological parameters (CTL, Tregs, MHC class I and II) have previously been reported by our group [18;37;38]. For the current study data on immunological parameters of the primary EOC sample subgroup (n = 270) were selected and correlated to the new WT1 immunostaining performed on the TMA.

TMAS

TMAs were constructed and described previously [37;39]. In brief, paraffin-embedded tissue blocks containing tumor in ovarian and omental tissue, and corresponding haematoxylin and eosin (H&E) stained slides were retrieved from the pathology archives. Representative areas of tumor were marked on the H&E slides. Next, using these H&E slides for reference four 0.6mm² core biopsies were taken from each tumor specimen and arrayed on a recipient paraffin block using a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). We were cautious to avoid taking cores from necrotic areas, large infiltrates and stromal tissue. Using a microtome, 4 µm sections were cut from each TMA block and applied to aminopropyltriethoxysilane-coated slides. All arrayed samples were H&E-stained to confirm the presence of tumor tissue.

IMMUNOHISTOCHEMICAL STAINING OF TMAS

TMA sections were de-paraffinized in xylene and rehydrated through graded concentrations of ethanol to destilled water. The sections were boiled for 15 minutes in a microwave oven to accomplish antigen retrieval. Endogenous peroxidase was blocked by incubation of sections for 30 minutes in 0.3% hydrogen peroxide.

TMA sections were stained for WT1 with clone 6F-H2 (DAKO, Glostrup, Denmark) in a dilution of 1:25. Antigen retrieval was done by Tris/HCL (pH9). Sections were counterstained with hematoxylin. Antibodies for immunostaining of immunological parameters were applied and reported in previous studies [18;37;38].

SCORING

Evaluation of WT1 immunostaining was independently performed by two observers blinded to the clinical data. Agreement between the two observers was >90%. Contradictory outcomes were reviewed by a gynecologic pathologist and were reassigned by approval of all parties. The immunohistochemical reaction for WT1 was interpreted semi-quantitatively by assessing the intensity

and extent of staining to a four-tiered (0 to 3) scale [9;10]. Nuclear immunoreactivity was considered positive. Negative staining is considered to correspond with normal WT1 expression, and positive staining represents WT1 overexpression. First the total percentage of positively stained tumor cells was determined and graded into four classes: negative = no immunopositive cells; + = low frequency (≤ 25%); ++ = moderate frequency (25%-50%); +++ = high frequency (50%-75%); ++++ = very high frequency (75-100%). Then the intensity was determined into four classes: none (0), weak (1), moderate (2) and strong (3). The cutoff was 'a priori' chosen for scoring, based on cutoff values used by others [9;10]: cases with nuclear immunoreactivity of any intensity and extent were considered positive for WT1 expression. Immunostaining for CTL, Treg, MHC class I and MHC class II was scored and reported previously by our group [18;37;38]. For the anti-CD8 staining, the number of cells with membrane staining within the tumor epithelium was counted for each core. For the anti-FoxP3 staining, the number of cells with nuclear staining within tumor-islets in every core was counted. Anti-CD8 and anti-FoxP3 staining was performed on consecutive slides of the TMA. To obtain a high concordance rate with whole tissue slides, we decided that minimally two cores with a minimum of 20% tumor tissue had to be present on the TMA for a sample to be entered into analysis [40]. Subsequently, we calculated the number of intra-tumoral cells per 0,238 mm^2 of tumor (i.e. one whole core consisting of 100% tumor tissue), to correct for differences in the amount of tumor tissue and to standardize the analysis. This calculation entailed dividing the total number of intra-tumoral cells in cores containing ≥20% of tumor by the total percentage of tumor tissue present in these cores. As a control we previously performed a pilot study on whole ovarian cancer slides in which numbers of CD8 and FoxP3 were equally present compared to our TMA (unpublished data). Cores containing stromal or necrotic tissue were excluded from analysis. All cases with <2 evaluable cores were excluded from analysis.

STATISTICAL ANALYSIS OF DATA

Statistical analysis was carried out using the SPSS 16.0 software package for Windows (SPSS Inc., Chicago, USA). To determine whether the EOC patient study cohort was representative, clinicopathological characteristics in all patients were evaluated (Table 1). WT1 overexpression (as dependent factor) was evaluated in relation to clinicopathological characteristics (Table 1) and immunological parameters (Table 2) (as independent factors) in both univariate and multivariate logistic regression models to characterize the WT1 vaccine target group. Factors with a *P* value of >0.10 in univariate analysis were excluded stepwise in multivariate analysis; in the final model, only factors with a *P* value of <0.05 were included. The distribution of anti-CD8 and anti-FoxP3 staining was positively skewed. For further analysis, we therefore decided to categorize patients using the bottom tertile (p33) as a cut-off value [41]. However, when the bottom tertile equaled zero, a subdivision based on the presence or absence of these cells was made for further analysis.

Progression free survival (PFS) was defined as time from primary surgery until progression/relapse of the disease or the date of last follow-up. Disease specific overall survival (OS) was defined as time from diagnosis until the last follow-up alive or death due to ovarian cancer. In the WT1 overexpressing subgroup, differences in PFS and OS according to clinicopathological characteristics (data not shown) and immunological parameters (Table 3) were analysed using both univariate and

Table 1 Clinicopathological characteristics all patients and WT1 overexpressing subgroup

	All patients (*n* = 270)	**WT1 overexpression (*n* = 129)**	**WT1 overexpression** *univariate*	**WT1 overexpression** *multivariate*
	n (%)	*n* (%)	**OR (95% CI)**	**OR (95% CI)**
FIGO[a] stage				
Early[b] stage	93 (34.6)	21 (16.3%)		
Advanced[c] stage	176 (65.4)	108 (83.7%)	**6.4 (3.5-11.9)**	**4.0 (1.9-8.6)**
Missing	1	0		
Tumor type				
Serous	147 (59.8)	97 (84.3%)	**11.3 (5.8-22.0)**	6.7 (3.2-13.6)
Non-serous[d]	99 (40.2)	18 (15.7%)		
Missing	24	14		
Tumor grade				
Low grade	128 (50.8)	44 (36.7%)		
High grade	124 (49.2)	76 (63.3%)	**2.9 (1.7-5.2)**	†
Missing	18	9		

[a]FIGO = International Federation of Gynaecology and Obstetrics. [b]Early stage: stage I and II. [c]Advanced stage: stage III and IV. [d]Non-serous: mucinous, endometrioid, clear cell and undifferentiated epithelial ovarian cancer.
†Not included in multivariate logistic regression analysis.
Bold signifies $P < 0.05$

multivariate Cox regression analyses to establish potential keystones for manipulation by new immunotherapeutic strategies. As a control, these Cox regression analyses have also been performed for all patients with EOC irrespective of WT1 status (data not shown). Variables with a *P* value of >0.10 in univariate analysis were excluded stepwise in multivariate analysis; in the final model, only factors with a *P* value of <0.05 were included.
Survival curves were generated using Kaplan-Meier method, with evaluation of the differences by the Mantel-Cox log-rank test. For all tests, *P* values <0.05 were considered statistically significant. All p-values were two-sided.

RESULTS

PATIENTS

From a total of 361 ovarian cancer patients, sufficient paraffin-embedded ovarian and/or omental tissue containing epithelial ovarian carcinoma was available for construction of the TMA. In 112 cases both primary ovarian tumor tissue and omental implants were available. From 158 patients we only have primary ovarian cancer material. For the current study, tumor samples from 270 (112 + 158) consecutive primary EOC patients (median age 56.9 years, range 16-89) treated at the UMCG between 1985 and 2006 were used. The majority of all patients presented with serous histology, advanced stage, and/or high grade disease (Table 1).

WT1 OVEREXPRESSION IN RELATION TO CLINICOPATHOLOGICAL CHARACTERISTICS

WT1 immunostaining could not be evaluated in 41/270 (15.2%) primary tumor samples due to core loss or absence of tumor tissue. WT1 overexpression was observed in 129/229 (56.3%) primary ovarian tumor samples (Table 2). Figure 1 shows a representative example of negative and positive WT1 immunostaining. WT1 overexpression was associated with advanced stage of disease [odds ratio (OR), 6.4; 95% confidence interval (95% CI), 3.5-11.9; $P < 0.001$; Table 1], serous type (OR, 11.3; 95% CI, 5.8-22.0; $P < 0.001$), and high grade (OR, 2.9; 95% CI, 1.7-5.2; $P < 0.001$). Independent predictors of WT1 overexpression were advanced stage (OR, 4.0; 95% CI; 1.9-8.6; $P < 0.001$) and serous type (OR, 6.7; 95% CI, 3.3-13.6; $P < 0.001$) (Table 1).

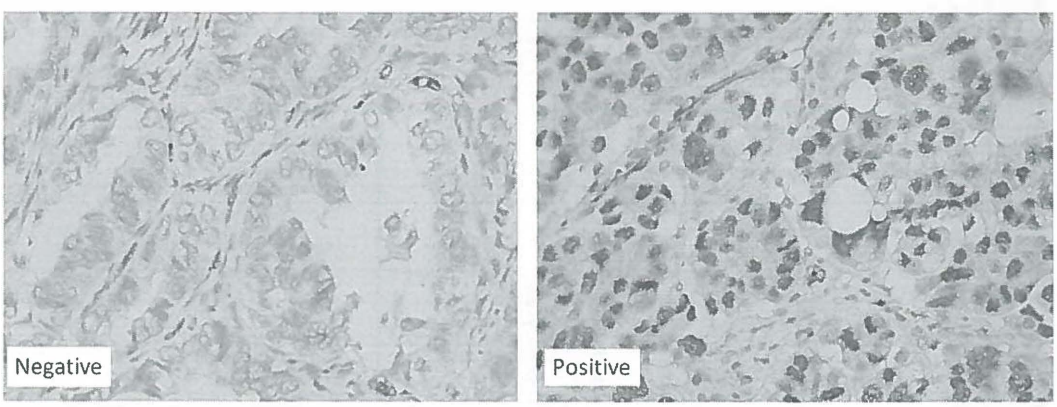

Figure 1 WT1-immunostaining epithelial ovarian cancer

Table 2 Relation between WT1 expression and immunostaining

All patients (*n* = 270)	**Normal, *n*/total (%)**	**Overexpression, *n*/total (%)**	**WT1 Overexpression present** *univariate*	**WT1 Overexpression present** *multivariate**
	n = 100[a] (43.7%)	*n* = 129[a] (56.3%)	**OR (95% CI)**	**OR (95% CI)**
High CD8$^+$ T-lymphocytes	59/98 (60.2%)	88/126 (69.8%)	1.5 (0.9-2.7)	†
Presence FoxP3$^+$ T-lymphocytes	37/98 (37.8%)	79/127 (62.2%)	**2.7 (1.6-4.7)**	‡
High CD8$^+$/FoxP3$^+$ ratio	59/83 (71.1%)	74/114 (64.9%)	0.8 (0.4-1.4)	†
Down-regulation MHC class I	39/98 (39.8%)	44/127 (34.6%)	0.8 (0.5-1.4)	†
Up-regulation MHC class II	35/77 (45.5%)	58/90 (64.4%)	**2.2 (1.2-4.1)**	†
Advanced serous subgroup (*n* = 127)				
	n = 23[b] (21.1%)	*n* = 86[b] (78.9%)	**OR (95% CI)**	**OR (95% CI)**
High CD8$^+$ T-lymphocytes	15/23 (65.2%)	56/83 (67.5%)	1.1 (0.4-2.9)	†
Presence FoxP3$^+$ T-lymphocytes	12/23 (52.2%)	50/84 (59.5%)	1.3 (0.5-3.4)	†
High CD8$^+$/FoxP3$^+$ ratio	17/22 (77.3%)	47/73 (64.4%)	0.5 (0.2-1.6)	†
Down-regulation MHC class I	9/23 (39.1%)	32/84 (38.1%)	1.0 (0.4-2.5)	†
Up-regulation MHC class II	14/18 (77.8%)	38/61 (62.3%)	0.5 (0.1-1.6)	†

[a]Missing: *n* = 41. [b]Missing: *n* = 18. †Not included in multivariate logistic regression analysis. ‡Not included in the final model of the multivariate analysis. *Multivariate analysis was corrected for clinicopathological characteristics age, stage, tumor type, tumor grade and residual disease (data not shown)

Bold signifies $P < 0.05$

IMMUNOLOGICAL PARAMETERS IN EOC WITH WT1 OVEREXPRESSION

High infiltration of $CD8^+$ CTL, any presence of $FoxP3^+$ Tregs, MHC class I down-regulation and MHC class II up-regulation were observed in 69.8%, 62.2%, 34.6% and 64.4% of WT1 overexpressed tumors, respectively (Table 2). Treg infiltration was more frequently observed in WT1 overexpressed tumors (OR, 2.7; 95% CI; 1.6-4.7; $P < 0.001$) in comparison to normally WT1 expressed tumors, in univariate logistic regression analysis. Also MHC class II was up-regulated more often in WT1 overexpressing EOC (OR, 2.2; 95%; 1.2-4.1; $P = 0.014$) (Table 2). As WT1 overexpression was associated with advanced stage of disease and serous tumor type, we also analyzed immunological parameter expression in the advanced stage serous subgroup of EOC. In advanced serous EOC high infiltration of $CD8^+$ CTL, any presence of $FoxP3^+$ Tregs, MHC class I down-regulation and MHC class II up-regulation were observed in 67.5%, 59.5%, 38.1% and 62.3% of WT1 overexpressed tumors, respectively (Table 2). None of the immunological parameters in the advanced serous EOC subgroup were correlated to WT1 overexpression.

SURVIVAL ANALYSIS IN (WT1 OVEREXPRESSING) EOC PATIENTS

When analyzing all EOC patients both well-known prognostic clinicopathological characteristics such as advanced stage disease, poorly differentiated tumors, serous tumors and ≥ 2cm residual disease, as well as immunological parameters $FoxP3^+$ and MHC class I down-regulation, were associated with a shorter OS and PFS, while high intraepithelial $CD8^+$ T-lymphocytes were associated with longer survival in EOC (data not shown). WT1 overexpression was also associated with a shorter OS and PFS [hazard ratio (HR), 2.1; 95% CI; 1.4-3.1; $P < 0.001$ and HR, 2.3; 95% CI; 1.6-3.4; $P < 0.001$, respectively]; illustrated by Kaplan Meier curves (Figure 2a, 2b).

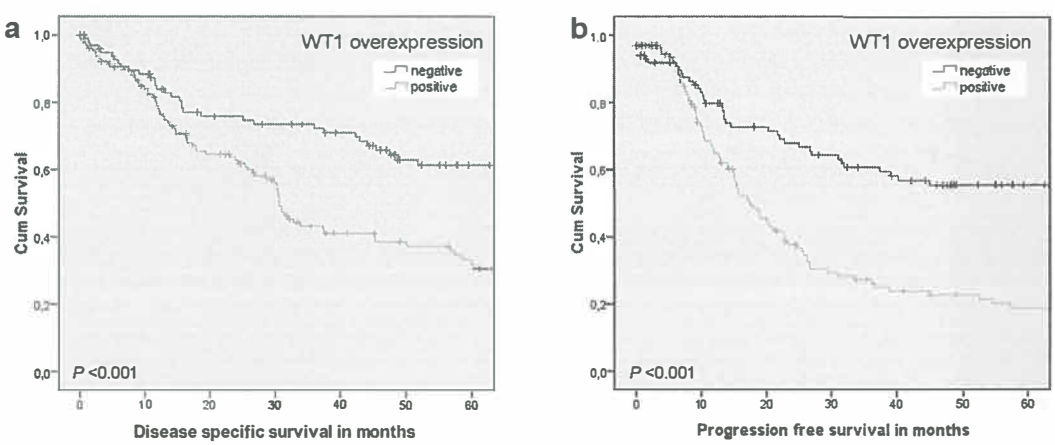

Figure 2 (a) Disease specific overall survival in all patients with negative or positive WT1 overexpression, $n = 229$ (84.8%), missing = 41 (15.2%). (b) Progression free survival in all patients with negative or positive WT1 overexpression, $n = 228$ (84.4%), missing = 42 (15.6%)

In the subgroup of patients with WT1 overexpression, high numbers of intra-epithelial CD8+ T-lymphocytes were associated with longer OS (HR, 0.6; 95% CI, 0.4-1.0; $P = 0.047$) and PFS (HR, 0.6; 95% CI, 0.4-0.9; $P = 0.020$) (Table 3), as illustrated by Kaplan Meier curves (Figure 3a, 3b). High numbers of CD8+ T-lymphocytes in WT1 overexpressing EOC is an independent prognostic factor for PFS (HR, 0.5; 95% CI, 0.3-0.8; $P = 0.006$) when corrected for clinicopathological characteristics age, stage, tumor type, tumor grade and residual disease (data not shown) (Table 3).

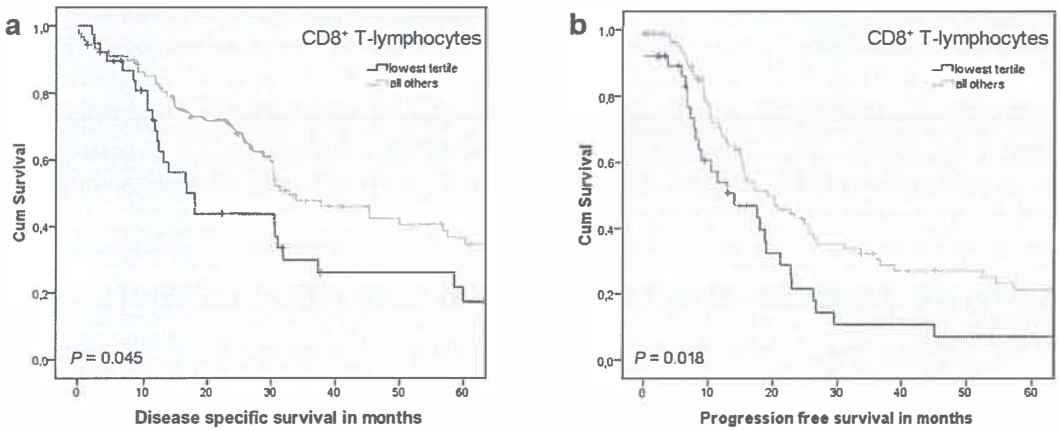

Figure 3 (a) Disease specific overall survival in WT1 overexpressing tumors with low or high CD8+ infiltration rates, $n = 126$ (97.7%), missing = 3 (2.3%). (b) Progression free survival in WT1 overexpressing tumors with low or high CD8+ infiltration rates, $n = 126$ (97.7%), missing = 3 (2.3%)

Table 3 Relation between immunological parameters and survival in WT1 overexpressing subgroup

Subgroup WT1 overexpression (*n* =129)	**Disease specific overall survival** *univariate*	**Progression free survival** *univariate*	**Disease specific overall survival** *multivariate*	**Progression free survival** *multivariate**
	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
High $CD8^+$ T-lymphocytes	**0.6 (0.4-1.0)**	**0.6 (0.4-0.9)**	†	**0.5 (0.3-0.8)**
Presence $FoxP3^+$ T-lymphocytes	0.8 (0.5-1.3)	1.1 (0.7-1.6)	†	†
High $CD8^+/FoxP3^+$ ratio	0.8 (0.4-1.1)	0.8 (0.5-1.2)	†	†
Down-regulation MHC class I	1.2 (0.7-1.9)	1.1 (0.7-1.8)	‡	†
Up-regulation MHC class II	1.2 (0.7-2.1)	1.2 (0.7-2.1)	†	†

†Not included in multivariate Cox regression analysis. ‡Not included in the final model of the multivariate analysis. * Multivariate analysis was corrected for clinicopathological characteristics age, stage, tumor type, tumor grade and residual disease (data not shown). Bold signifies $P < 0.05$

DISCUSSION

WT1 is a promising immunotherapeutic target, but lacks clinical efficacy when administered in a therapeutic vaccine. We performed a detailed study to clarify the association of WT1 and immunological parameters in a large, well documented series of EOC patients to generate new clues how to modify vaccination strategies to improve immunologic and clinical efficacy. Our data suggest that in EOC with WT1 overexpression, high numbers of intra-epithelial $CD8^+$ CTL are associated with improved survival. Simultaneously EOC with WT1 overexpression is more likely to be infiltrated with regulatory T-cells and up-regulated with MHC class II, while MHC class I downregulation was observed in one-third of WT1 overexpressing EOC. These observations point to the design of novel immunotherapeutic strategies targeting WT1 in combination with depletion of Tregs and up-regulation of MHC class I. Such an approach might enhance the clinical effect induced by a WT1-vaccine, especially for treatment of advanced stage, serous type EOC.

Our results confirm the observation that WT1 overexpression is more common in high-grade, advanced stage, serous ovarian cancer [9-11] which supports the idea for a vaccine targeting WT1 for treatment of this high risk EOC patient subgroup. Although WT1 initially was categorized as tumor-suppressor gene, it was demonstrated that the wild-type WT1 gene has an oncogenic rather than a tumor-suppressor function in malignancies [42;43]. Overexpression of WT1 was associated with poor OS and PFS. This is in agreement with the idea that WT1 is involved in the acquisition of invasiveness by mesenchymal-epithelial transition observed in early ovarian tumorigenesis [42;44]. A theoretical advantage of using WT1 protein as a target antigen is its essential role in tumorigenesis. Consequently, escape from immune surveillance by down-regulation of WT1 expression will have a negative influence on tumor growth [6]. Several clinical trials using WT1 targeting vaccines were initiated based on promising preclinical results [29;45;46]. Solid cancer patients treated with WT1 peptide based vaccines demonstrated WT1-related immunological responses, but clinical responses failed to materialize [31-33;35]. This limited clinical efficacy therefore, dictates further exploration of new immunization strategies.

Positivity for WT1 staining in primary EOC was based on 'a priori' chosen immunohistochemical scoring method and cutoff value, based on methods used by others [9;10]. This cutoff value has its limitations, as a detailed semi-quantitatively scoring method was used, but WT1 immunostaining was considered positive, regardless of intensity, class or score. However, analysis of WT1 expression in a qualitative manner showed no correlation to any immunological parameter and survival.

Our finding of high intra-epithelial $CD8^+$ CTL numbers being associated with improved survival in WT1 overexpressing EOC, does not prove these CTL being WT1 specific or even active for that matter. Future clinical trials on WT1 vaccines should focus on analysis of tumor-infiltrating T cells before and after WT1 immunization, with particular interest in whether the vaccine-induced T cells are WT1 specific. However, our finding of high CTL numbers having a better prognosis supports

the hypothesis that enhancement of the number of T cells, whether or not WT1 specific, after a WT1 based vaccination might benefit the patient.

One WT1 specific immunotherapeutic approach is by vaccination with synthetic long peptides (SLPs), spanning the complete sequence of the WT1 protein [47;48]. SLP-vaccination may contain multiple T-helper and CTL epitopes, which broaden the immune responses induced while reducing MHC restrictions as all potential MHC class I and MHC class II epitopes within the delivered peptides will be processed and presented to host T-cells [49]. Clinical efficacy and vaccine induced immunity using a SLP vaccine was observed by Kenter *et al.* who tested a SLP vaccine against the HPV-16 oncoproteins E6 and E7 in patients with HPV-16 positive, grade 3 vulvar intraepithelial neoplasia [48].

MHC class I down-regulation was observed in 34.6% of WT1 expressing EOC tumors. Down-regulation of MHC class I, as one of the known tumor immune escape mechanisms, may partly explain the poor prognosis of WT1 overexpressing tumors. Considering the importance of MHC class I expression by tumor cells for immune recognition by CTL, several regimens could be incorporated in a WT1 based vaccination strategy to enhance MHC class I expression. Treatment with IFN-gamma is known to up-regulate MHC class I [50-52]. Another possibility would be addition of demethylating agents to the WT1 vaccine, since DNA hyper-methylation, common in human tumors, may result in the loss of MHC class I expression [53;54].

Presence of Tregs seems more profound in EOC overexpressing WT1 and was also correlated to advanced stage, serous type, and high grade EOC (data not shown), as was partly found by others [21;22]. The observed correlation between WT1 overexpression and Treg infiltration might be due to the tendency of WT1 positive tumors to be advanced stage and serous type rather than independently correlated. On the other hand was the presence of Tregs in WT1 overexpressing EOC correlated to MHC class II up-regulation (OR, 3.4; 95% CI, 1.4-8.3; $P = 0.009$). Moreover, on WT1 overexpressing EOC also more MHC class II was up-regulated compared to EOC cells with normal WT1 expression. As MHC class II can present tumor associated-antigens to $CD4^+$ T-helper cells which contribute to an anti-tumor CTL response MHC class II up-regulation would have been likely to result in improved survival, as was reported in ovarian cancer and other cancer types [27;55-58]. Our lack of association with survival could be explained by the fact that MHC class II complexes also can present tumor-associated antigens to immune inhibitory $CD4^+FoxP3^+$ Tregs. Our findings suggest that in EOC WT1 might be presented by MHC class II on its tumor cell surface attracting $CD4^+FoxP3^+$ Tregs, resulting in the poor prognosis of WT1 overexpressing EOC. However, like Tregs MHC class II expression was also correlated to advanced stage, serous EOC (data not shown), as was found by others [59]. So the poor prognosis could also be explained by the fact that serous, advanced EOC are more likely to show WT1 overexpression, Treg infiltration and MHC class II up-regulation. Immunosuppression mediated by Tregs is a major obstacle for successful tumor immunotherapy as Tregs suppress antigen specific T cell responses [60-63]. Strategies to eliminate or suppress Tregs to improve clinical efficacy of immunotherapy vary from treatment with low dose cyclophosphamide, fludarabine or COX-2 inhibitors, or using antibodies against CD25, blocking CTLA-4 or through TGF-β blockade [64-71].

Our study group previously performed a clinical trial on an SLP-vaccine targeting p53 in advanced stage EOC patients [47]. We observed that the p53-SLP vaccine is able to enhance the number of p53-specific $CD4^+$ T-helper (Th) cells, whereas no p53-specific CTLs are induced. This was to be expected as the p53-specific $CD8^+$ T cell repertoire but not the $CD4^+$ T cell repertoire is severely restricted by self-tolerance and might only consist of lower-affinity p53-specific $CD8^+$ T cells [72;73]. Optimal interplay between Th-cells and CTLs is required to induce a robust cell-mediated antitumor immunity. To optimize the effect of the SLP vaccine CTL induction alongside the Th cells is required. Future studies on the p53-SLP vaccine combined with WT1 might be promising, as this tumor antigen is able to induce WT1-specific $CD8^+$ T cells [32;33;35]. This p53 /WT1-SLP vaccine combined with immunopotentiating regimens could ultimately result in successful anti-tumor response and consequently lead to clinical response.

In summary, high $CD8^+$ CTL infiltration of EOC overexpressing WT1 is associated with increased survival. WT1 overexpression is more commonly observed in serous, advanced stage EOC and therefore is a promising target for immunotherapeutic treatment of this high risk subgroup of EOC patients. Vaccines targeting WT1 could be combined with strategies to e.g. eliminate Tregs and/or up-regulate MHC class I, and/or by combination with T-helper cells inducing tumor antigen vaccines, such as p53-SLP, in an attempt to induce clinical efficacy.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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CHAPTER 7

PROGRAMMED DEATH-1 LIGANDS ARE EXPRESSED IN THE MAJORITY OF ADVANCED SEROUS EPITHELIAL OVARIAN CANCER BUT HAVE NO PROGNOSTIC IMPACT

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ABSTRACT

Characterization of immune effector cells and immune escape mechanisms at the primary tumor site enables the design of more effective immunotherapeutic strategies in epithelial ovarian cancer (EOC). Therefore we determined the prognostic importance of immune inhibitory programmed death-1 ligands 1 (PD-L1) and 2 (PD-L2) in 127 tumor samples of a well-defined homogeneous group of primary advanced serous EOC patients. Moreover, PD-L1/L2 expression was correlated with the number of tumor infiltrating lymphocytes (TIL) and TIL-subsets data, i.e. $CD8^+$ cytotoxic- (CTL), $FoxP3^+$ regulatory- (Tregs) and $CD45R0^+$ memory T-cells. Clinicopathological characteristics, follow-up, and TIL-subset data, were derived from a previously published dataset. PD-L1 and PD-L2 expression was observed in 65 (61.9%) and 77 (73.3%) of 127 tumor samples, respectively. Survival was not influenced by expression of PD-L1 or PD-L2 in advanced serous EOC. Moreover, PD-L1 expression did not correlate with the number of one of the TIL subsets. PD-L2 expression correlated negatively with the number of memory T-cells [odds ratio (OR), 0.2; 95% confidence interval (95% CI), 0.1-0.5; $P = 0.001$] and positively with the number of CTL (OR, 3.2; 95% CI, 1.1-9.5, $P = 0.037$). PD-L1 and PD-L2 expression do not seem to influence the activity of TILs, as no prognostic influence was observed in each TIL subset by PD-Ls.

In conclusion, despite high expression rates of PD-L1/L2, PD-Ls have no prognostic impact in advanced serous EOC.

INTRODUCTION

Due to a lack of specific symptoms most epithelial ovarian cancer (EOC) patients are diagnosed with advanced stage of disease [1;2]. Despite aggressive treatment with cytoreductive surgery and platinum-taxane based chemotherapy tumor recurrences often occur, resulting in a poor overall survival [3;4]. Therefore, new therapeutic strategies are urgently required. Since the presence of immune effector cells positively predict the clinical course of EOC patients, immunotherapeutic strategies are considered promising innovative treatment modalities in EOC [5].

Immunization strategies targeting various single tumor-associated antigens (TAAs) have been developed and tested in ovarian carcinoma patients, but no single approach has proven clinically effective yet [6;7]. Disappointing clinical results were explained by immune escape mechanisms and suppression of antigen-specific immunity by tumor cells and the tumor microenvironment [8;9]. Several immune escape mechanisms have been identified such as T-cell anergy through insufficient B7 co-stimulation, extrinsic suppression by regulatory T-cell populations, inhibition by co-stimulatory molecule cytotoxic T-lymphocyte antigen-4, metabolic deregulation by enzymes like indoleamine-2,3-dioxygenase, and inhibitory factors such as TGF-β [10;11].

A recently discovered immune escape mechanism is the immune suppressive pathway of the programmed death-1 (PD-1) receptor, an immune inhibitory receptor predominantly expressed on activated T-cells [12;13]. PD-1 and its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) play a critical role in T-cell regulation [14-17]. PD-L1 is expressed on T- and B-cells, macrophages and dendritic cells, and regulates activation and differentiation of T-cells mediating peripheral tolerance [18]. PD-L1 is also abundantly expressed in various human cancers, including oesophageal, pancreatic, hepatocellular and ovarian carcinomas [19-22]. Tumor-associated PD-L1 increases apoptosis of antigen-specific T-cells contributing to the failure of cytotoxic T-lymphocyte (CTL) mediated TAA-specific immunity in cancer patients, leading to tumor growth in vivo [6].

Conflicting results on the prognostic effect of PD-L1/L2 expression on tumor cells are reported [19-26]. Moreover, there is little information on the importance of PD-L1/L2 expression by tumor cells in relation to tumor infiltrating T-lymphocytes (TILs) in ovarian cancer patients. Ovarian cancer is a heterogeneous disease in which histological phenotypes correlate with distinct genetic events [27;28]. Most deaths are attributable to the serous tumor type, which comprises approximately 60% of cases and has a propensity to present at an advanced stage of disease [3]. We performed a detailed study determining the prognostic effect of PD-L1/L2 expression in tumor samples of a well-documented, homogeneous cohort of primary advanced serous EOC patients. We also evaluated PD-L1/L2 expression in correlation with the number of tumor infiltrating CTL, regulatory T-cells (Tregs), and memory T-cells. The data might contribute to the rational design of innovative immunotherapeutic strategies for EOC.

MATERIALS AND METHODS

PATIENTS

Since 1985 the Department of Gynecological Oncology of the University Medical Center Groningen (UMCG) prospectively stored all clinicopathologic and follow-up data of EOC patients in a computerized database. Samples of ovarian tumor tissue from 361 patients were collected on a tissue microarray (TMA) among which 270 tumor samples obtained at primary surgery prior to chemotherapy (period 1985-2006), selected for the current study. Data on clinicopathologic factors, follow-up, survival and TILs (CTL, Tregs and memory T-cells) have previously been reported by our group [29]. For the current study data on TILs of primary advanced serous EOC samples (*n* = 127) were selected and correlated to the new PD-L1 and PD-L2 immunostaining performed on the TMA.

TMAS

TMAs were constructed as described previously [29-31]. Four cores of 0.6mm² were taken by biopsy and placed by a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA) on a recipient paraffin block. Using a microtome, 4 µm sections were cut from each TMA block and applied to aminopropyltriethoxysilane-coated slides. All arrayed samples were hematoxylin and eosin stained to confirm the presence of tumor tissue.

IMMUNOHISTOCHEMICAL STAINING OF TMAS

TMA sections were de-paraffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water. Antigen retrieval was accomplished by microwave oven treatment in citrate buffer pH 6.0 for PD-L1 and EDTA buffer pH 8.0 for PD-L2, respectively. Endogenous peroxidase was blocked by incubation of sections for 30 minutes in 0.3% hydrogen peroxide.
TMA sections were stained for PD-L1 with a rabbit polyclonal antibody (B7-H1 protein, CD274, Lifespan biosciences, Seattle, USA) in a dilution of 1:300 and for PD-L2 a mouse monoclonal antibody (clone XX19, Santa Cruz Biotechnology, Heidelberg, Germany) was used in a dilution of 1:50. Sections were subsequently incubated with DAKO Envision+ (DAKO, Glostrup, Denmark) for PD-L1 and rabbit-anti-mouse/HRP (DAKO) followed by goat-anti-rabbit/HRP (DAKO) for PD-L2. Antigen-antibody reactions were visualized with 3, 3'Diaminobenzidine (DAB) and the sections were counterstained with hematoxylin. All control experiments gave satisfactory results. Antibodies for immunostaining of TILs were applied and reported in a previous study [29].

SCORING

Evaluation of PD-L1 and PD-L2 immunostaining was independently performed by two observers blinded to the clinical data. Agreement between the two observers was >90%. Contradictory outcomes were reviewed by a gynecological pathologist and were reassigned by approval of all parties. The immunohistochemical reaction for PD-L1 and PD-L2 was interpreted by assessing the

intensity of staining to a four-tiered (0 to 3) scale [22]. Membranous immunoreactivity was considered positive. The intensity was determined into four classes: none (0), weak (1), moderate (2), and strong (3). The cutoff was 'a priori' chosen for scoring: cases with scores 0 or 1 were considered negative for PD-L1 and PD-L2 expression, respectively, and cases with scores 2 and 3 were considered positive. Immunostaining for CTLs, Tregs and memory T-cells was scored and reported previously by our group [29]. For analysis of TIL subtypes the tumors were categorized using the bottom tertile (p33) as a cutoff value [32]. All cases with <2 evaluable cores were excluded from analysis.

STATISTICAL ANALYSIS OF DATA

Statistical analysis was carried out using the PASW statistics 18 software package for Windows (PASW Inc., Chicago, USA). To determine whether the EOC patient study cohort was representative, clinicopathological characteristics and survival in all primary EOC patients were evaluated (Table 1). Disease specific overall survival (OS) was defined as time from diagnosis to death due to ovarian cancer or last follow-up visit alive. Progression free survival (PFS) was defined as time from primary surgery until progression/relapse of the disease or the date of last follow-up.

The cut off value for all TIL subsets is described in our previous study [29]. Expression of PD-L1/L2 and presence of TILs and its three main subsets CTL, Tregs and memory T-cells in advanced serous EOC were depicted to illustrate our study cohort (Table 2). Next to analysis of CTL, Tregs and memory T-cells the total number of TILs and ratio of CTL/Tregs was analyzed (Table 2).

In the advanced serous EOC patients, differences in PFS and OS according to PD-L1/L2 expression, TILs and clinicopathological characteristics were analyzed using both univariate and multivariate Cox regression analyses (Table 3). Factors with a *P* value of >0.10 in univariate analysis were excluded stepwise in multivariate analysis; in the final model, only factors with a *P* value of <0.05 were included.

PD-L1 and PD-L2 expression (as dependent factor) were evaluated in relation to TILs (as independent factors) in both univariate and multivariate logistic regression models in advanced serous EOC to determine the inhibitory effect of PD-L1 and/or PD-L2 on the number of TILs (Table 4).

To determine the influence of PD-L1 and PD-L2 expression on the activity of TILs we determined differences in PFS and OS in each TIL subset, individually (Table 5). Variables with a *P* value of >0.10 in univariate Cox regression analysis were excluded stepwise in multivariate analysis; in the final model, only factors with a *P* value of <0.05 were included.

Survival curves were generated using Kaplan-Meier method, with evaluation of the differences by the Mantel-Cox log-rank test. For all tests, *P* values <0.05 were considered statistically significant. All *P* values were two-sided.

Table 1 Tumor characteristics of all EOC patients and advanced serous EOC patients

	All patients (*n* = 270)	**Advanced serous patients (*n* = 127)**
	n (%)	*n* (%)
FIGO[1] *stage*		
Early[2] stage	93 (34.6)	0
Advanced[3] stage	176 (65.4)	127 (100.0)
Missing	1	0
Tumor type		
Serous	147 (59.8)	127 (100.0)
Non-serous[4]	99 (40.2)	0
Missing	24	0
Tumor grade		
Low grade	128 (50.8)	42 (35.3)
High grade	124 (49.2)	77 (64.7)
Missing	18	8
Residual disease		
<2 cm	155 (62.2)	46 (39.3)
≥2 cm	94 (37.8)	71 (60.7)
Missing	21	10
Disease specific survival		
Median[5]	17	16
Range	0-167	0-167
5-year survival	45.6%	20.4%
Progression free survival		
Median[5]	13	11
Range	0-160	0-160
5-year survival	4.5%	3.1%
Follow-up		
Median[5]	35	19
Range	0-275	0-252

[1]FIGO = International Federation of Gynaecology and Obstetrics. [2]Early stage: stage I and II. [3]Advanced stage: stage III and IV. [4]Non-serous: mucinous, endometrioid, clear cell and undifferentiated epithelial ovarian cancer. [5]In months.

Table 2 PD-L1/L2 and tumor infiltrating T-lymphocytes in advanced serous EOC

	Advanced serous patients[1] (*n* = 127)
	n (%)
PD-L1	
Negative	40 (38.2)
Positive	65 (61.9)
Missing	22
PD-L2	
Negative	28 (26.7)
Positive	77 (73.3)
Missing	22
Tumor infiltrating T-lymphocytes	
Lowest tertile	44 (38.6)
All others	70 (61.4)
Missing	13
$CD8^+$ T-lymphocytes	
Lowest tertile	39 (33.6)
All others	77 (66.4)
Missing	11
$FoxP3^+$ T-lymphocytes	
Absent	55 (45.5)
Present	66 (54.5)
Missing	6
$CD45R0^+$ T-lymphocytes	
Absent	66 (55.9)
Present	52 (44.1)
Missing	9
$CD8^+/FoxP3^+$ ratio	
Lowest tertile	33 (32.7)
All others	68 (67.3)
Missing	26

[1]Advanced stage: stage III and IV, according to FIGO (International Federation of Gynaecology and Obstetrics)

Table 3 Relation between PD-L1/L2, immunological and clinicopathological parameters, and survival in advanced serous EOC

Advanced serous patients[1] (*n* =127)	**Disease specific overall survival** *univariate*	**Progression free survival** *univariate*	**Disease specific overall survival** *multivariate*	**Progression free survival** *multivariate*
	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
PD-L1 positive	0.9 (0.6-1.4)	0.9 (0.6-1.4)	†	†
PD-L2 positive	1.1 (0.7-1.8)	1.1 (0.7-1.8)	†	†
High tumor infiltrating T-lymphocytes	**0.6 (0.4-0.9)**	0.7 (0.4-1.1)	**0.4 (0.3-0.7)**	†
High $CD8^+$ T-lymphocytes	**0.6 (0.4-0.9)**	**0.6 (0.4-1.0)**	†	**0.5 (0.3-0.8)**
Presence $FoxP3^+$ T-lymphocytes	0.8 (0.6-1.3)	1.0 (0.7-1.6)	†	†
Presence $CD45R0^+$ T-lymphocytes	0.8 (0.5-1.2)	1.0 (0.6-1.5)	†	†
High $CD8^+/FoxP3^+$ ratio	0.7 (0.4-1.0)	0.7 (0.4-1.1)	†	†
Age ≥58 years	1.2 (0.8-1.7)	1.3 (0.8-1.9)	†	†
High grade	**1.8 (1.2-2.8)**	1.1 (0.7-1.6)	**2.1 (1.6-3.5)**	†
Residual disease ≥2cm	2.1 (1.4-3.2)	2.5 (1.6-3.9)	2.3 (1.4-3.6)	2.8 (1.7-4.5)

[1]Advanced stage: stage III and IV, according to FIGO (International Federation of Gynaecology and Obstetrics)
†Not included in multivariate Cox-regression analysis. Bold signifies $P < 0.05$

Table 4 Relation between PD-L1/PD-L2 expression and tumor infiltrating T-lymphocytes

	PD-L1 expression			
Advanced serous patients[1] (*n* =127)	**Negative, *n*/total**	**Positive, *n*/total**	**PD-L1 positive** *univariate*	**PD-L1 positive** *multivariate**
	n = 40* (38.1%)	*n* = 65* (61.9%)	**OR (95% CI)**	**OR (95% CI)**
High tumor infiltrating T-lymphocytes	23/38 (60.5%)	42/63 (66.7%)	1.3 (0.6-3.0)	†
High $CD8^+$ T-lymphocytes	27/39 (69.2%)	44/63 (69.8%)	1.0 (0.4-2.5)	†
Presence $FoxP3^+$ T-lymphocytes	22/38 (57.9%)	39/65 (60.0%)	1.1 (0.5-2.5)	†
Presence $CD45R0^+$ T-lymphocytes	18/39 (46.2%)	29/64 (45.3%)	1.0 (0.4-2.1)	†
High $CD8^+/FoxP3^+$ ratio	25/37 (67.6%)	38/53 (71.7%)	1.2 (0.5-3.0)	†
	PD-L2 expression			
	Negative, *n*/total	**Positive, *n*/total**	**PD-L2 positive** *univariate*	**PD-L2 positive** *multivariate**
	n = 28** (26.7%)	*n* = 77** (73.3%)	**OR (95% CI)**	**OR (95% CI)**
High tumor infiltrating T-lymphocytes	16/27 (59.3%)	48/74 (64.9%)	1.3 (0.5-3.1)	†
High $CD8^+$ T-lymphocytes	16/27 (59.3%)	54/75 (72.0%)	1.8 (0.7-4.4)	**3.2 (1.1-9.5)**
Presence $FoxP3^+$ T-lymphocytes	15/27 (55.6%)	45/76 (59.2%)	1.2 (0.5-2.8)	†
Presence $CD45R0^+$ T-lymphocytes	19/27 (70.4%)	27/76 (35.5%)	**0.2 (0.1-0.6)**	**0.2 (0.1-0.5)**
High $CD8^+/FoxP3^+$ ratio	18/27 (66.7%)	45/64 (70.3%)	1.2 (0.5-3.1)	†

[1]Advanced stage: stage III and IV, according to FIGO (International Federation of Gynaecology and Obstetrics)

*/** Missing: *n* = 22 and *n* = 22, respectively. †Not included in multivariate logistic regression analysis. *Multivariate analysis was corrected for clinicopathological characteristics age, tumor grade and residual disease (data not shown). Bold signifies $P < 0.05$

Table 5 Relation between PD-L1 or PD-L2 expression and survival in immunological parameter subgroups of advanced serous EOC

	Disease specific overall survival *univariate*		**Progression free survival** *univariate*	
Subgroup	**PD-L1 positive expression**	**PD-L2 positive expression**	**PD-L1 positive expression**	**PD-L2 positive expression**
	HR (95% CI)	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**
High tumor infiltrating T-lymphocytes (n = 70)	0.8 (0.5-1.5)	1.3 (0.7-2.5)	0.8 (0.5-1.5)	1.1 (0.6-2.0)
High $CD8^+$ T-lymphocytes (n = 77)	0.9 (0.5-1.5)	1.1 (0.6-2.1)	0.9 (0.5-1.5)	1.0 (0.5-1.9)
Presence $FoxP3^+$ T-lymphocytes (n = 66)	1.0 (0.5-1.7)	1.7 (0.8-3.4)	0.9 (0.5-1.7)	1.4 (0.7-2.7)
High $CD8^+/FoxP3^+$ ratio (n = 68)	0.8 (0.5-1.5)	1.1 (0.6-2.1)	0.8 (0.5-1.5)	1.0 (0.5-1.8)
Presence CD $45R0^+$ T-lymphocytes (n = 52)	0.7 (0.3-1.3)	1.0 (0.5-1.9)	0.6 (0.3-1.2)	1.0 (0.5-1.9)

Bold signifies $P < 0.05$

RESULTS

PATIENTS

Tumor samples from 270 consecutive primary EOC patients (median age 56.9 years, range 16-89) treated at the UMCG between 1985 and 2006 were available. The majority of all patients presented with advanced stage of disease, and serous tumor type (n = 127) (Table 1). The majority of advanced serous EOC patients had high grade disease and presence of residual disease after primary debulking surgery.

PD-L1 AND PD-L2 EXPRESSION IN ADVANCED SEROUS EOC

Both PD-L1 and PD-L2 immunostaining could not be evaluated in 22 out of 127 (17.3%) primary advanced stage serous tumor samples, due to core loss or absence of tumor tissue (Table 2). PD-L1 and PD-L2 expression was observed in 65/105 (61.9%) and 77/105 (73.3%) of primary EOC samples, respectively. In advanced serous EOC high numbers of TILs (70/114 (61.4%)) were present, including high numbers of CTL and Tregs in 77/116 (66.4%) and 66/121 (54.5%) of tumor samples, respectively. Also a high CTL/Treg ratio was observed (68/101 (67.3%)). Memory T-cells were observed in less then half of advanced serous EOC (52/118 (44.1%)).

SURVIVAL ANALYSIS IN PRIMARY ADVANCED SEROUS EOC PATIENTS

PD-L1 and PD-L2 expression were not associated with OS [PD-L1: hazard ratio (HR), 0.9; 95% CI, 0.6-1.4, P = 0.641; PD-L2: HR, 1.1; 95% CI, 0.7-1.8; P = 0.761) and PFS (PD-L1: HR, 0.9; 95% CI, 0.6-1.4, P = 0.619; PD-L2: HR, 1.1; 95% CI, 0.7-1.8; P = 0.719; Table 3) in primary advanced serous EOC, as illustrated for OS by Kaplan Meier curves (Figure 1a, 1b). In advanced serous EOC high numbers of TILs were associated with longer OS (P = 0.010; Table 3). High numbers of intraepithelial CD8$^+$ CTL were associated with longer OS, as well as PFS (P = 0.012 and P = 0.057, respectively; Table 3). High numbers of TIL and CTL were independent prognostic factors for OS (TIL: P < 0.001) and PFS (CTL: P = 0.005; Table 3) in advanced serous EOC, respectively, illustrated

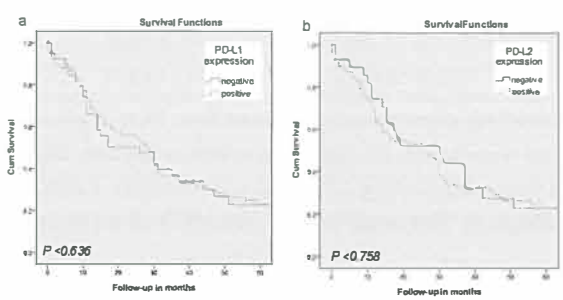

Figure 1 a) Disease specific overall survival PD-L1 expression in advanced serous EOC. b) Disease specific overall survival PD-L2 expression in advanced serous EOC.

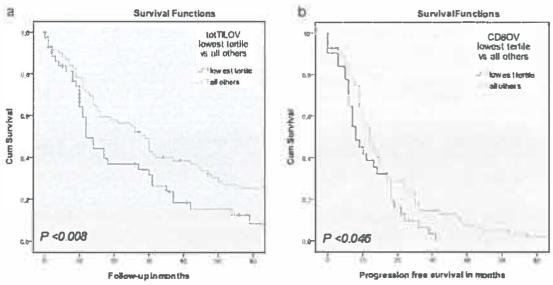

Figure 2 a) Disease specific overall survival TIL in advanced serous EOC. b) Progression free survival CTL infiltration in advanced serous EOC.

by Kaplan Meier curves (Figure 2a, 2b). Both high grade disease and residual tumor which are well-known prognostic factors in advanced serous EOC, were independently associated with OS (P = 0.002 and P = 0.001, respectively; Table 3). Residual disease is also a prognostic factor for PFS in advanced serous EOC (P < 0.001).

IMMUNOLOGICAL PARAMETERS IN EOC WITH PD-L1 OR PD-L2 EXPRESSION

High numbers of TILs, $CD8^+$ CTL, $FoxP3^+$ Tregs and $CD45R0^+$ memory T-cells, and high $CD8^+$/$FoxP3^+$ ratio were observed in 66.7%, 69.8%, 60.0%, 45.3%, and 71.7% of PD-L1 and in 64.9%, 72.0%, 59.2%, 35.5%, and 70.3% of PD-L2 expressing tumors, respectively (Table 4). PD-L1 expression of the advanced stage serous ovarian cancer cells was not correlated with the number of CTL, Tregs, and memory T-cells when compared to tumor cells with no PD-L1 expression. In contrast, PD-L2 expression on advanced stage EOC had an inhibitory effect on the number of memory T-cells [odds ratio (OR), 0.2; 95% confidence interval (95% CI), 0.1-0.5; P = 0.001; Table 4], independent of any other subset of TILs. Moreover, PD-L2 positive tumor cells tend to be infiltrated with a higher number of CTL (OR, 3.2; 95% CI, 1.1-9.5, P = 0.037), independent of clinicopathological characteristics and other TILs.

SURVIVAL ANALYSIS IN TIL SUBGROUPS OF ADVANCED STAGE SEROUS EOC

PD-L1 might inhibit the functionality of TILs, inducing apoptosis in PD-1 positive TILs. Therefore, the influence of PD-L1 presence or absence on survival in TIL subgroups was analyzed. PD-L1 seems to have no influence on "the activity" of tumor infiltrating CTL, Tregs, or memory T-cells using survival as a surrogate read-out parameter (Table 5). The same holds true for PD-L2 expression.

DISCUSSION

The prognostic value of PD-L1 expression on the tumor cell surface has been studied for several malignancies and was found to be associated with poor prognosis in pancreatic cancer, urothelial cancer, gastric carcinoma, renal cell carcinoma and ovarian cancer [20;22-24;33]. However, inconsistent findings regarding PD-L1 and prognosis have been observed in a variety of other tumors, including small-cell lung cancer and cervical cancer [25;26], therefore the prognostic relevance of PD-L1 expression remains controversial. PD-L1 had no influence on prognosis in our homogeneous well-documented cohort of advanced serous EOC.

The lack of prognostic association of PD-L1 in EOC in our study contrasts the results of a similar study in ovarian cancer patients of Hamanishi *et al.* [22]. This inconsistency could be due to different staining methods. We stained TMA sections for PD-L1 using a commercially available rabbit polyclonal antibody. Hamanishi *et al.* used a murine monoclonal antibody, which they generated specifically for this purpose. Another explanation is the difference in study cohort composition. The cohort we used for the PD-L1/L2 analysis consisted of a homogeneous patient group with advanced serous EOC. The cohort studied by Hamanishi *et al.* included a heterogeneous group of patients with ovarian cancers of different histological subtypes and stages. To make a true comparison we analyzed our heterogeneous group of 270 primary EOC for prognostic influence of PD-L1 and PD-L2. Nevertheless, the lack of association remained, as PD-L1 and PD-L2 expression were not associated with OS [PD-L1: hazard ratio (HR), 1.2; 95% CI, 0.5-1.8, $P = 0.278$; PD-L2: HR, 1.0; 95% CI, 0.7-1.5; $P = 0.847$) and PFS (PD-L1: HR, 1.0; 95% CI, 0.7-1.4, $P = 0.784$; PD-L2: HR, 1.2; 95% CI, 0.8-1.7; $P = 0.445$), respectively. Lastly, the inconsistent finding can possibly be explained merely on the relatively small cohort ($n = 70$) analyzed by Hamanishi *et al.* The cohort we analyzed consisted of 127 patients (homogeneous tumors) and 270 patients (heterogeneous tumors).

Different research groups observed a similar effect of PD-L1 expression on the number of infiltrating CTL, Tregs, and memory T-cells [12;14;34]. In contrast to these results we observed no correlation between tumor infiltrating T-lymphocytes and PD-L1 expression.

For PD-L2 expression, numerous research groups observed contrasting results about the function of PD-L2 on effector T-cells [35]. We observed that PD-L2 negatively correlated with the number of infiltrating memory T-lymphocytes and positively correlated with the presence of CTL in our study cohort. Yet, PD-L2 expression did not correlate favorably with prognosis. Ohigashi *et al.* reported an inverse correlation between PD-L2 status and $CD8^+$ TILs [19]. In addition, others found an inhibitory effect of PD-L2 on T-cell proliferation [36;37]. Contrastingly, Liu *et al.* showed that PD-L2 promotes tumor immunity independently of PD-1 [38]. PD-L2 reactivity independent of PD-1 was also observed in the regulation of the asthmatic response [39]. PD-L2 may not only influence responses by modifying T-cell receptor signaling, but also may deliver positive signals into PD-L2-expressing DCs [16;40;41]. Ellis *et al.* reported that specific types of antigen presenting cells, such as DCs display expression of PD-L2 supporting transition of effector T-cells into memory T-cells [42]. The contrasting results considering PD-L2 and TIL imply a considerable complexity of the PD-1/PD-L pathway and the possible unknown receptors that interact with PD-L. Therefore, additional pre-

clinical studies on the PD-1/PD-L pathway and careful interpretation will be required. Moreover, further research is warranted on the role of PD-L2 and its interaction with T-cell subsets.

We observed no influence of PD-L1 and PD-L2 expression on survival in subsets of TIL. A possible explanation for this lack of correlation with survival could be ascribed to a potential absence of PD-1 on the TILs. Regretfully, we were not successful to determine the expression of PD-1 on TILs using our TMA despite the use of antibodies that, according to the manufacturer, should stain PD-1 on paraffin embedded tissue.
The observed high PD-L2 expression rate in this study differs to the rates observed in ovarian-, lung- and esophageal cancer, as reported by others [19;22;25]. A possible explanation for these inconsistent findings on PD-L2 expression may be the small number of patients analyzed by others, the use of different antibodies to stain PD-L2, different staining protocols, and the use of fresh frozen versus paraffin-embedded material.

PD-1-blockade represents a potential strategy to inhibit the PD-1/PD-L pathway [12;13]. Anti-PD-1 antibodies combined with cancer vaccines may augment immunogenicity and clinical efficacy in cancer patients. Therapeutic combinations of anti-PD-1 with peptide-pulsed DC vaccines or toll-like receptor agonists CpG, have been tested successfully in mouse models [43;44]. So far, PD-1-blockade seems to have clinical activity in phase I clinical trials both in patients with refractory solid tumors and in patients with advanced hematologic malignancies [45-47]. Brahmer *et al* [45], recently demonstrated that tumor cell surface expression of PD-L1 is a predictor of responsiveness to PD-1-blockade. The high expression of PD-L1 in EOC observed in the current study, would suggest that also EOC could be a target for PD-1 blockade. Moreover, PD-1-blockade can reverse the inhibiting signal of PD-1 to both PD-L1 and PD-L2 expressing cells [40] therefore, the observed high PD-L2 expression on tumor cells might contribute to the response to anti-PD-1. Next, anti-PD-1 is described to have clear effects on reversing suppression of PD-1 expressing Tregs and also results in down-regulation of intracellular FoxP3 expression by Tregs [34;48]. Thus, the positive effect of PD-1 blockade on tumor growth could evolve from inactivation of immune inhibitory Tregs.

In summary, our data indicate that PD-1 blockade might be promising because of the high expression rates of PD-Ls in EOC. However, we need to be precautious considering the lack of correlation between PD-Ls and survival. Moreover, the observed lack of correlation between PD-L1 expression on the number of TIL in our well documented, homogeneous, large study cohort of advanced serous EOC, argues against PD-1 blockade. Further studies on PD-L2 function are warranted. Based upon our results, applicability of anti-PD-1 in EOC to augment immunogenicity and clinical efficacy of immunotherapy in advanced serous EOC seems controversial.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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CHAPTER 8

SUMMARY AND FUTURE PERSPECTIVES

SUMMARY

Epithelial ovarian cancer (EOC) is the most common cause of death in gynaecologic malignancies. Due to a lack of specific symptoms most EOC patients are diagnosed with advanced stage of disease. The majority of patients with advanced stage EOC achieve a complete clinical response due to the current therapy of aggressive cytoreductive surgery and platinum-taxane based chemotherapy. Unfortunately, most patients develop tumor recurrence, resulting in five-year survival rates of only 30%. Therefore, new therapeutic strategies are urgently required. Since the prognosis of EOC patients is influenced by tumor infiltrating immune effector cells [1-3], an immunotherapeutic approach to treat ovarian cancer seems promising. Immunization strategies targeting various single tumor-associated antigens have been developed and tested in EOC patients. In most studies these vaccines induce antigen-specific immunity, but substantial clinical efficacy has not been observed. This thesis describes the results of two clinical trials on an antigen-specific vaccine targeting tumor-antigen p53 in ovarian cancer patients with recurrent disease. Next, we studied intratumoral parameters in EOC in an effort to increase our knowledge on how to improve the clinical efficacy of antigen-specific vaccines in EOC patients.

A short introduction on cancer immunotherapy is provided in **chapter 1**. This chapter highlights the background of ovarian cancer and tumor immunology, and illustrates the possibilities for clinical implementation of (antigen-specific) immunotherapy.

In the following three chapters the use of p53 as a target for antigen-specific vaccines in cancer patients is described. P53, a tumor-suppressor protein, is a potential target for cancer immunotherapy as mutations in the p53 gene are frequently seen in human oncogenesis. Persistent overexpression of p53 is present in 50-60% of ovarian cancers. Therefore, the majority of ovarian cancer patients might benefit from p53-directed immunotherapy. Previously, a phase I/II clinical trial has been performed to study the immunogenicity and clinical efficacy of a p53-specific synthetic long peptide (SLP) vaccine in recurrent ovarian cancer patients [4]. Induction of p53-specific T-cells, but limited clinical efficacy was observed in the study cohort.
We were interested if treatment with secondary chemotherapy after treatment with the p53-SLP vaccine influences the long-term memory of the p53-specific immune response. Therefore, we conducted a follow-up study in 8 recurrent ovarian cancer patients initially treated with the p53-SLP vaccine in our phase I/II clinical trial, described in **chapter 2**. Antonia *et al.* reported that vaccine-induced p53-specific immune responses were associated with improved response to secondary chemotherapy in small cell lung cancer patients [5]. Treatment with the p53-SLP vaccine in our study cohort of ovarian cancer patients, did not affect responses to secondary chemotherapy or survival. However, p53-specific T-cells could still be retrieved despite the use of chemotherapy. From our previous phase I/II clinical trial and the current follow-up study we can conclude that the p53-SLP vaccine is able to induce both short- and long-term immunity.
In our previous phase I/II clinical trial we observed limited clinical effect induced by the p53-SLP vaccine. This could possibly be explained by the presence of regulatory T-cells (Tregs) which are

known to suppress immune responses. We hypothesized that low-dose cyclophosphamide, which is described to selectively deplete Tregs, might be able to enhance the effect of the p53-SLP vaccine. Therefore we performed a phase II clinical trial in recurrent ovarian cancer patients combining low-dose cyclophosphamide, a chemotherapeutic agent, with the p53-SLP vaccine **(chapter 3)**. The p53-SLP vaccine preceded by cyclophosphamide induced p53-specific T-cells. It also resulted in p53-specific T-cell proliferation, which coincided with the production of T-helper 1 and T-helper 2 cytokines. Depletion of Tregs however was not observed, both in number and activity. Comparing the results of the current phase II clinical trial with the previous phase I/II clinical trial, a stronger induction of p53-specific T-cells was observed. Furthermore, the reduction in number of circulating p53-specific T-cells observed in our previous phase I/II study after four immunizations was absent in the current phase II study. The results of the current study support the use of low-dose cyclophosphamide potentiating immunogenicity of cancer vaccines, such as vaccines targeting p53 in ovarian cancer patients.

As p53 is an obvious target for cancer immunotherapy, several studies on p53-based vaccines for immunotherapeutic treatment of cancer patients have been conducted over the past decade. In **chapter 4** we summarize clinical trials performed so far using a p53-based vaccine in different cancer types. Vaccination strategies varying from viral vectors, dendritic cells, short and long peptides have been used. Although peptide-loaded DC and long peptides have induced reasonably strong p53-specific immune responses, in particular $CD4^+$ T-helper cell responses, robust clinical responses have failed to materialize.

From chapter 2, 3 and 4, we can conclude that p53-vaccines are able to induce p53-specific immune responses, predominantly $CD4^+$ T-helper cells. The limited clinical efficacy upon immunization with the current p53 vaccines warrants further exploration of innovative immunization strategies.

In the following three chapters we describe several parameters studied in EOC in an effort to increase our knowledge on how to improve the clinical efficacy of antigen-specific vaccines in EOC patients.

Whereas solid tumors often show heterogeneous protein expression, immunization using a cocktail of antigens may have greater therapeutic potential compared to single targeted vaccines. Targeting multiple antigens may also improve the immunogenicity of the vaccine thereby increasing its clinical efficacy. We determined the expression of SP17 and NY-ESO-1 and overexpression of p53, WT1 and survivin next to MHC class I expression in 270 primary EOC patients **(chapter 5)**. Over 90% of EOC express at least one of the investigated tumor antigens. These results indicate that a multi-epitope vaccine, comprising these antigens, could serve as a universal therapeutic vaccine for the vast majority of ovarian cancer patients.

A new potential tumor antigen in EOC is WT1, as it is able to induce $CD8^+$ specific cytotoxic T-cells (CTL) in patients with mesothelioma and non-small cell lung cancer [6]. Analysis of immunological parameters at the primary tumor site of EOC in the context of WT1 expression might provide new clues how to modify vaccination strategies trying to improve immunogenicity and clinical efficacy. In **chapter 6** we therefore analyzed the presence of CTLs, Tregs, MHC class I, and class II expression

next to the overexpression of WT1 in 270 primary EOC patients. High numbers of intra-epithelial $CD8^+$ CTL are associated with improved survival in EOC overexpressing WT1. However, EOC with WT1 overexpression is more likely to be infiltrated with Tregs and up-regulated with MHC class II, while MHC class I down-regulation was observed in one-third of WT1 overexpressing EOC. These observations point to the design of an immunotherapeutic strategy targeting WT1 in combination with depletion of Tregs and up-regulation of MHC class I.

Analysis of immune evading strategies at the primary tumor site might be helpful in designing strategies to disrupt this immune inhibition. A recently discovered immune escape mechanism is the immune suppressive pathway of the programmed death-1 (PD-1) receptor, and its ligands PD-L1/L2. Anti-PD-1 antibodies inhibiting the PD-1/PD-L1 pathway combined with cancer vaccines might augment immunogenicity and clinical efficacy in cancer patients. Contrasting results on the prognostic effect of PD-L1/PD-L2 expression in tumor cells are reported. Therefore we determine the prognostic value of PD-L1/L2 expression in a homogeneous, well-documented cohort of 127 primary advanced serous ovarian cancer patients **(chapter 7)**. Little information is available on the importance of PD-L1/L2 expression by tumor cells in relation to TIL in ovarian cancer patients. Consequently, we analyzed the influence of PD-L1/L2 expression on tumor infiltration of cytotoxic-, memory-, and regulatory T-lymphocytes. Survival was not influenced by expression of PD-L1 or PD-L2 in advanced serous EOC. Moreover, PD-L1 expression did not correlate with the number of one of the TIL subsets. PD-L2 expression correlated negatively with the number of memory T-cells and positively with the number of CTL. PD-L1 and PD-L2 expression do not seem to influence the activity of TILs, as no prognostic influence was observed in each TIL subset by PD-Ls. In conclusion, despite high expression rates of PD-L1/L2, PD-Ls have no prognostic impact in advanced serous EOC. Our data indicate that PD-1 blockade seems promising because of the high expression rates of PD-Ls in EOC, but the lack of influence of PD-Ls on survival and number of TILs, argue against it.

CONCLUSIONS

In this thesis we describe two clinical vaccination trials targeting p53 in ovarian cancer patients. Moreover, we describe the immunogenicity and clinical efficacy observed in clinical trials on p53-vaccines, performed by others. Next, we studied parameters in EOC in an effort to increase our knowledge on how to improve the clinical efficacy of immunotherapeutic strategies in EOC patients.

The p53-SLP vaccine is a promising cancer vaccine as it is able to induce both short-term and long-term p53-specific immunity. Moreover, cyclophosphamide is a promising agent to potentiate the p53-SLP vaccine in recurrent ovarian cancer patients. Up until now, p53-vaccines in general have been shown to induce p53-specific immunity, in particular $CD4^+$ T-helper cells, rather than a clinical effect in cancer patients. A possible approach to augment the immunogenicity and clinical effect of a p53-vaccine is by addition of other antigens such as SP17, NY-ESO-1, WT1 and survivin, aiming to successfully treat EOC patients. WT1 could also be targeted alone, if combined with Treg depletion and up-regulation of MHC class I. PD-1 receptor blockade aiming to augment the effect of

antigen-specific vaccines, such as p53-SLP, is controversial as no prognostic influence of its ligands PD-L1/L2 was observed in EOC patients.

FUTURE PERSPECTIVES

Although active immunotherapy seems to be a potent strategy to establish clinical benefit in ovarian cancer patients, this optimism has been tempered by the fact that ovarian cancer, as Chiriva-Internati *et al.* stated, represents a paradigm for the art of immunological defense [7]. The pathogenesis of ovarian cancer is characterized by multiple immunosuppressive mechanisms in the tumor microenvironment, several of which correlate with increased morbidity and mortality in ovarian cancer patients. Understanding the immunology of ovarian cancer, with a particular emphasis on the tumor microenvironment will likely guide the development of novel multimodality treatment strategies to result in clinically effective ovarian cancer vaccination.

A recent discovered member of the $CD4^+$ T-helper response is T-helper 17 (Th17). Kryczek *et al.*, analyzed the tumor-infiltrating $CD4^+$ T-cell population from ovarian cancer samples for the presence of Th17 cells [8]. Ovarian cancers with a higher number of tumor infiltrating $CD4^+$ T-cells expressing interleukin-17 (IL-17), indicative of Th17 differentiation, had a significantly better overall survival, irrespective of their tumor stage. Th17 cells were found to be associated with pro-inflammatory cytokines and chemokines, and inversely correlated with the number of tumor infiltrating suppressive Tregs. It is shown that macrophages isolated from ovarian tumors biased the in vitro differentiation of uncommitted $CD4^+$ T-cells toward Th17 cells, indicating differing conditions within different tumors, favoring either inflammation (Th17) or suppression (Tregs). The inverse relationship between Th17 cells and Tregs observed in ovarian cancers may be more than simply a descriptive association, as suggested by several studies. It may reflect fundamental differences in the nature of the spontaneous antitumor immune response, differences that appear to have significant impact on patient survival.
The precise role of Th17 responses in tumor immunity and pathogenesis has drawn some lively discussion of apparently conflicting results, since Th17 cells have also been associated with disease progression in various experimental models. However, for ovarian cancer there is a strong case to be made for further investigation of the potential therapeutic benefits of vaccination strategies designed to boost Th17 responses. Knowledge on how to tip the balance between negative signals resulting in Treg-associated immune suppression, and positive signals that promote Th17-associated antitumor immunity, may unlock the door to successful treatment.

Angiogenesis is a critical step for the development and metastasis of virtually all cancers. Vascular endothelial growth factor (VEGF) stimulates angiogenesis. Thus, VEGF and its receptors are promising therapeutic targets. A phase II clinical trial evaluated the use of the anti-VEGF monoclonal antibody, bevacizumab, for persistent or recurrent EOC with promising clinical results [9]. Thalidomide also displays anti-angiogenic properties, through blockade of basic fibroblast growth factor-

induced and VEGF-induced angiogenesis, and modulates the immune system by inducing IFN- , IL-2, and IL-10. A phase II clinical trial including women with recurrent EOC showed a higher response rate to thalidomide compared to conventional therapy [10]. However, a more recent phase III study did not confirm these results and reported significantly higher thalidomide related toxicity [11].

Indoleamine 2,3-dioxygenase (IDO) catalyzes tryptophan degradation, and its activity results in inhibited T-cell proliferation due to tryptophan depletion. Elevated expression of IDO creates a tolerogenic environment in the tumor site and in the tumor-draining lymph nodes, suppressing effector T-cells and promoting Treg function. Immune suppression mediated by IDO expression may be alleviated by treatment with a small molecule competitive inhibitor of enzyme activity, 1-methyl tryptophan (1-MT), which is currently being tested in clinical trials [12].

Bevacizumab, thalidomide, and 1-MT are agents that need to be evaluated more extensively as adjuvant treatment for successful ovarian cancer immunotherapy. In that regard the precise role of VEGF and its receptors, and IDO in the tumor microenvironment of ovarian cancer needs to be clarified to support the design of clinically effective ovarian cancer vaccination. Next to VEGF and IDO a plethora of other mechanisms of the tumor micro-environment must be evaluated to increase the knowledge of the tumor immunology of ovarian cancer.

A limited clinical effect is observed in our patient cohort of recurrent ovarian cancer upon immunization with the p53-SLP vaccine. In future clinical studies, timing of immunization could be reconsidered as advanced cancer patients generally have large and/or metastatic tumors which are difficult to treat with immunotherapy [13]. Large tumors have been shown to have multiple, often redundant, pathways of immune escape that causes advanced cancer patients to be poor candidates for immunotherapeutic vaccines, such as p53-SLP.
Perhaps the strongest case for the p53-SLP vaccine would be if it is administered as an adjuvant treatment strategy in ovarian cancer patients who have undergone standard therapy, consisting of surgery and chemotherapy. The concept that chemotherapy in general results in a strong reduction of major components of the immune system, thereby hampering anti-tumor immunity, does not hold true anymore. Cisplatinum next to paclitaxel and doxorubicin, drugs often used in ovarian cancer, make tumor cells more susceptible to Granzyme B dependent killing by CTL [14]. Moreover, the calreticulin exposure pathway is postulated to be an important mechanism of activation of the immune system after treatment with classical therapies like chemotherapy [15]. Immunotherapy in combination with chemotherapy might be a very effective strategy as induction of long lived antigen-specific memory T-cells recently have been identified [16]. Furthermore, the interaction of tumor cell death due to chemotherapy on one hand, and induction of anti-tumor immune responses induced by this cell death on the other hand, might be essential to achieve the optimal result in tumor eradication [17-19].
In future studies it could therefore be advisable to test the p53-SLP vaccine as adjuvant therapy, combined with the current aggressive therapy of cytoreductive surgery and platinum-taxane based chemotherapy in ovarian cancer patients.

In summary, new insights in antigen-specific immunotherapy and combination strategies to augment its function, for successful treatment of EOC patients, are provided in this thesis. The clinical effect induced by these immunotherapeutic strategies however, remains limited. In the near future, both preclinical and clinical studies will undoubtedly shed more light on the possibilities of combined immunotherapeutic strategies to successfully treat EOC patients. Knowledge on the impact of ovarian tumor development and progression on the immune system will form a cornerstone in the development of a multimodality approach for successful treatment of ovarian cancer.

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CHAPTER 9

NEDERLANDSE SAMENVATTING

INLEIDING

In Nederland wordt jaarlijks bij 1200 vrouwen eierstokkanker vastgesteld. En jaarlijks overlijden 1000 vrouwen aan deze vorm van kanker. We ontdekken eierstokkanker vaak in een laat stadium. De meeste symptomen van de ziekte treden namelijk pas op als de kanker zich heeft uitgebreid in de buikholte. Bij deze patiënten is opereren alléén meestal niet voldoende. Daarom worden deze patiënten ook met chemotherapie behandeld. Meestal reageert de tumor in eerste instantie goed op deze behandeling. Toch krijgt het merendeel van de patiënten binnen twee jaar opnieuw kanker. Vaak zijn de kankercellen dan echter minder gevoelig voor chemotherapie. Dit betekent dat 5 jaar na het vaststellen van de diagnose slechts 30% van de patiënten nog leeft. Deze sombere overlevingscijfers vragen om nieuwe behandelmethoden. In dit proefschrift laten we zien dat immunotherapie daarvoor in aanmerking komt. Immunotherapie is een behandelmethode waarbij we door middel van vaccinaties het immuunsysteem stimuleren om kankercellen te vernietigen.

Het immuunsysteem bestaat uit twee onderdelen: het aangeboren en het aangeleerde immuunsysteem. In dit proefschrift beperken we ons tot het aangeleerde immuunsysteem. Het aangeleerde immuunsysteem bevat verschillende cellen die van belang zijn bij de afweer tegen kankercellen, waaronder cytotoxische T-cellen, T-helper-cellen en regulatoire T-cellen. Cytotoxische T-cellen kunnen kankercellen vernietigen doordat zij tumor-specifieke antigenen herkennen op het oppervlak van een kankercel. Daartoe moeten ze worden gestimuleerd door T-helper-cellen. Regulatoire T-cellen hebben daarentegen een remmend effect op de werking van cytotoxische- en T-helper-cellen. De tumor-specifieke antigenen worden op het oppervlak van de kankercel gepresenteerd door het zogenaamde 'Major Histocompatibility Complex' (MHC) molecuul. Cytotoxische T-cellen herkennen tumorantigenen die worden gepresenteerd door MHC-klasse I moleculen. T-helper-cellen en regulatoire T-cellen herkennen tumorantigeen dat door MHC-klasse II gepresenteerd wordt. Het fraaie van het aangeleerde immuunsysteem is dat het in staat is geheugen op te bouwen tegen een specifiek tumorantigeen door middel van geheugen-T-cellen.

Met immunotherapie proberen we door middel van een kankervaccin de natuurlijke afweerreactie van het lichaam tegen kankercellen dusdanig te versterken dat het immuunsysteem kankercellen herkent en vernietigt. In het geval van eierstokkanker zijn verschillende vaccins getest. Het blijkt dat deze vaccins wel in staat zijn om antigeen-specifieke cytotoxische T-cellen en T-helper-cellen op te wekken, maar dat zij de tumorgroei niet remmen. Een verklaring voor de geringe klinische effectiviteit ligt misschien in de mechanismen die kankercellen gebruiken om aan afweerreacties te ontsnappen. Meer kennis van deze mechanismen is dus belangrijk. We kunnen de werking van kankervaccins misschien verbeteren als we deze vaccins combineren met een behandeling die de ontsnappingspoging van de kankercel onderdrukt. Deze combinatietherapie zou uiteindelijk tot een beter klinisch resultaat moeten leiden.

In dit proefschrift reiken we nieuwe aanknopingspunten aan om de werking van kankervaccins te verbeteren.

SAMENVATTING VAN DIT PROEFSCHRIFT

In de eerste 3 hoofdstukken van het proefschrift beschrijven we het gebruik van een kankervaccin dat is gebaseerd op het p53-eiwit. P53 is een eiwit dat tot expressie komt in normale cellen. Het eiwit is echter in sterk verhoogde mate aanwezig in de kankercel van ruim de helft van de patiënten met eierstokkanker. Omdat kankercellen veel meer p53 bevatten dan normale cellen kan het p53 eiwit een tumorantigeen genoemd worden.
In een eerdere klinische studie hebben we een vaccin getest dat bestaat uit lange stukjes (peptiden) van het p53-eiwit. Dit synthetische lange peptide (SLP) vaccin, p53-SLP-vaccin, is getest in patiënten met eierstokkanker. Het vaccin is veilig, wordt goed verdragen en kan een p53-specifieke afweerreactie teweeg brengen. De patiënten waren eerder behandeld voor eierstokkanker, maar hadden op het moment van deelname aan de studie opnieuw kanker (een recidief) zonder klachten.

Wanneer patiënten met een recidief van eierstokkanker wel klachten krijgen worden ze opnieuw met chemotherapie behandeld. We waren benieuwd of de chemotherapie het lange termijn geheugen van de p53-specifieke afweerreactie van onze studiepatiënten zou beïnvloeden. Ook vroegen we ons af of de gevoeligheid van de kankercellen voor chemotherapie vergroot zou zijn door de aanwezigheid van p53-specifieke T-cellen. In **hoofdstuk 2** laten we zien dat er nog steeds p53-specifieke T-cellen te meten zijn, ook nadat patiënten opnieuw met chemotherapie zijn behandeld. De respons van de kankercellen op de chemotherapie was echter niet verbeterd door de aanwezigheid van een p53-specifieke afweerreactie. Kennelijk is het p53-SLP-vaccin goed in staat om zowel op de korte als op de lange termijn een p53-specifieke afweerreactie te bewerkstelligen. Zoals gezegd is het p53-SLP-vaccin in staat een p53-specifieke afweerreactie te realiseren; het vaccin bleek echter niet klinisch effectief. Een mogelijke verklaring is de aanwezigheid van regulatoire T-cellen. Deze cellen hebben een remmend effect op het immuunsysteem. Er zijn aanwijzingen dat regulatoire T-cellen specifiek geremd kunnen worden door een lage dosis chemotherapie (bijvoorbeeld cyclofosfamide). In **hoofdstuk 3** worden de resultaten beschreven van een klinische studie waarbij we het p53-SLP-vaccin in combinatie met cyclofosfamide testten in patiënten met een recidief eierstokkanker. De toevoeging van cyclofosfamide resulteerde niet in een verandering van het aantal en de functie van regulatoire T-cellen. Het resulteerde echter wel in een zeer sterke toename van het aantal p53-specifieke T-helper-cellen in vergelijking met de eerste studie waarbij patiënten alleen met het vaccin waren behandeld. Dit resultaat bevestigt dat de toevoeging van cyclofosfamide de werking van een kankervaccin kan verbeteren. Hoe dat kan, moet nog verder uitgezocht worden.
In de afgelopen 10 jaar hebben meerdere onderzoeksgroepen een p53-vaccin getest in kankerpatiënten. In **hoofdstuk 4** wordt een overzicht gegeven van alle klinische studies die tot dusver zijn uitgevoerd met een p53-vaccin. Dit overzicht bestaat niet alleen uit studies uitgevoerd in patiënten met eierstokkanker, maar ook in patiënten met andere soorten kanker. Uit deze studies blijkt dat behandeling met een p53-vaccin weliswaar een p53-specifieke afweerreactie kan veroorzaken in kankerpatiënten, maar nog geen klinisch effect heeft.

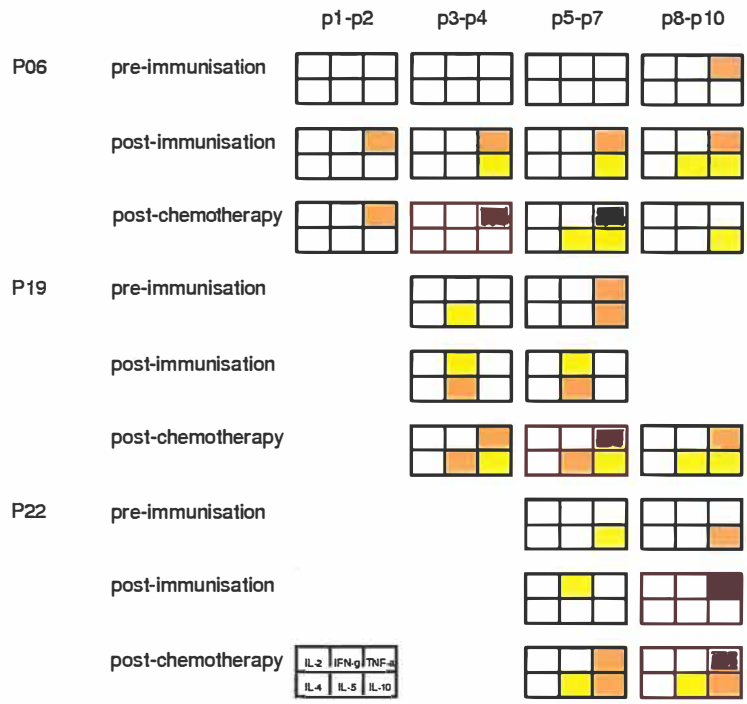
In de volgende 3 hoofdstukken van het proefschrift analyseren we enkele onderdelen van het immuunsysteem die in verband staan met eierstokkanker. De verkregen kennis kan gebruikt worden om de effectiviteit van kankervaccins te vergroten.

Zoals eerder genoemd komt p53 bij ongeveer de helft van de patiënten met eierstokkanker tot overexpressie. Kankercellen kunnen meerdere tumorantigenen tot expressie brengen. Daarom zou een kankervaccin dat naast p53 uit verschillende andere tumorantigenen bestaat effectiever kunnen zijn. In **hoofdstuk 5** is naast de expressie van p53, ook gekeken naar de expressie van de tumorantigenen SP17, NY-ESO-1, WT1 en survivin bij 270 patiënten met eierstokkanker. Het blijkt dat bij meer dan 90% van de patiënten, één of meer van de onderzochte tumorantigenen tot expressie komt. Dit resultaat doet vermoeden dat een vaccin bestaande uit deze 5 antigenen als een kankervaccin zou kunnen dienen voor verreweg de meerderheid van de patiënten met eierstokkanker.
Een nieuw, veelbelovend, tumorantigeen dat in eierstokkanker tot overexpressie komt, is WT1. WT1 komt ook in normale cellen tot expressie maar is in kankercellen in veel hogere mate aanwezig. **Hoofdstuk 6** beschrijft onderzoek naar de aanwezigheid van cytotoxische T-cellen, regulatoire T-cellen, MHC klasse I en II, en overexpressie van WT1 in 270 patiënten met eierstokkanker. Het blijkt dat patiënten bij wie de kankercellen veel cytotoxische T-cellen bevatten en WT1-overexpressie hebben, een betere kans op overleving hebben. Tumoren met WT1-overexpressie bevatten echter ook meer regulatoire T-cellen. Daarnaast hebben deze tumoren minder MHC klasse I op het celoppervlak. Daarom zou een WT1-vaccin mogelijk efficiënter zijn als we het combineren met een behandeling waarbij regulatoire T-cellen worden onderdrukt en de aanwezigheid van MHC klasse I wordt gestimuleerd.
PD-1, een onlangs ontdekte receptor, kan een afweerreactie onderdrukken als het een verbinding aangaat met PD-L1 of PD-L2. PD-L1 is in staat om celdood van antigeenspecifieke T-cellen te veroorzaken. Zo draagt het bij aan het falen van een cytotoxische T-cel respons. Blokkade van de verbinding tussen PD-1 en PD-L1/L2 schakelt de remmende werking op het immuunsysteem uit.
In **hoofdstuk 7** beschrijven we de invloed van PD-L1 en PD-L2-expressie op de overleving in een cohort van 127 patiënten met eierstokkanker in een vergevorderd stadium. De verwachting is dat de aanwezigheid van PD-L1 of L2 een negatief effect heeft op de overleving omdat het de aanwezigheid van T-cellen doet verminderen en daarmee de afweerreactie tegen kankercellen onderdrukt. We hebben de invloed van PD-L1/L2 expressie op de aanwezigheid van cytotoxische-, geheugen-, en regulatoire T-cellen onderzocht. De meerderheid van de patiënten met eierstokkanker in een vergevorderd stadium heeft PD-L1 en/of PD-L2 expressie, maar PD-L1 en PD-L2 hebben geen (negatieve) invloed op de overleving. Bovendien heeft de aanwezigheid van PD-L1 geen invloed op het aantal T-cellen. Ook lijkt PD-L1 de werking van T-cellen in de kankercel niet te beïnvloeden. We zouden dus kunnen concluderen dat het nuttig is om de PD-1-PD-L1/L2 verbinding te blokkeren omdat PD-L1 en PD-L2 bij zoveel patiënten met eierstokkanker tot expressie komt. Het gebrek aan invloed op overleving en T-cellen pleit echter tegen het gebruik van deze blokkade. Wat precies de rol is van PD-1 en PD-L1/L2 in eierstokkanker moet nog onderzocht worden. Dat kan bijvoorbeeld door de aanwezigheid van PD-1 op de T-cellen te analyseren.

SAMENVATTING

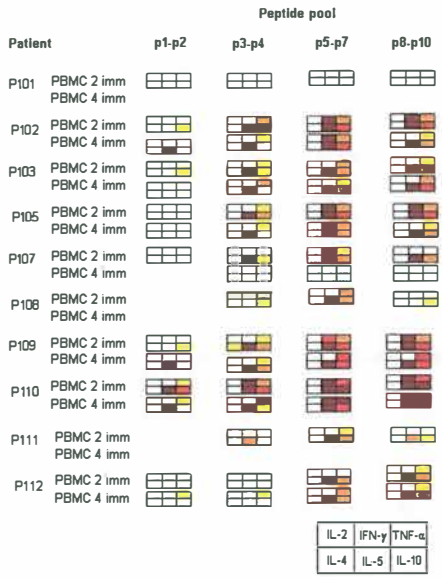
In dit proefschrift zijn aanknopingspunten gegeven om de behandeling van eierstokkanker te verbeteren. Uit het onderzoek blijkt dat het p53-SLP-vaccin in immunologisch opzicht een veelbelovend vaccin is dat zowel een korte- als een lange termijn p53-specifiek T-cel-geheugen kan veroorzaken. De werking van het p53-SLP-vaccin kan worden verbeterd door het te combineren met cyclofosfamide. Verder is gebleken dat met een kankervaccin waarin p53 gecombineerd is met meerdere tumorantigenen, zoals SP17, NY-ESO-1, WT1 en survivin, mogelijk de meerderheid van de patiënten met eierstokkanker behandeld zou kunnen worden. WT1 zou in een kankervaccin gebruikt kunnen worden. Een combinatie van een WT1 vaccin met een therapie waarbij regulatoire T-cellen worden onderdrukt en/of aanwezigheid van MHC klasse I wordt gestimuleerd lijkt wenselijk. De meerderheid van de patiënten met eierstokkanker in een vergevorderd stadium heeft PD-L1 en/of PD-L2 expressie, maar PD-L1 en PD-L2 hebben geen (negatieve) invloed op de overleving. In de toekomst zullen de resultaten van zowel preklinisch als klinisch onderzoek meer duidelijkheid geven over de therapieën waarmee kankervaccins het beste gecombineerd kunnen worden om de behandeling van eierstokkanker te verbeteren. Kennis over de samenhang tussen eierstokkanker en het immuunsysteem vormt de basis voor de verdere ontwikkeling van een succesvolle behandeling voor eierstokkanker.

APPENDIX: COLOR FIGURES



Chapter 2, **Figure 3**

Chapter 2, **Figure 4**



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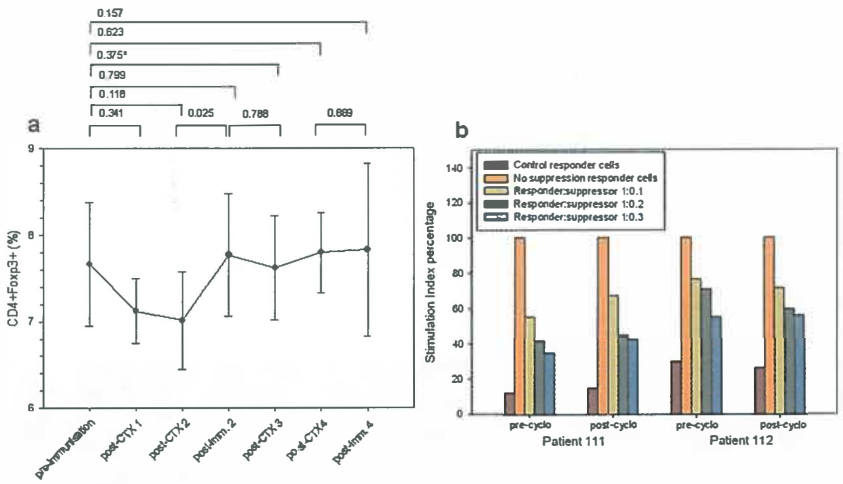
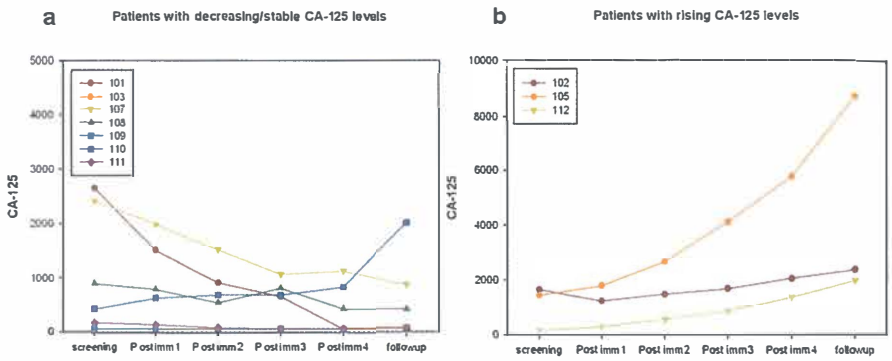


Chapter 3, **Figure 3**



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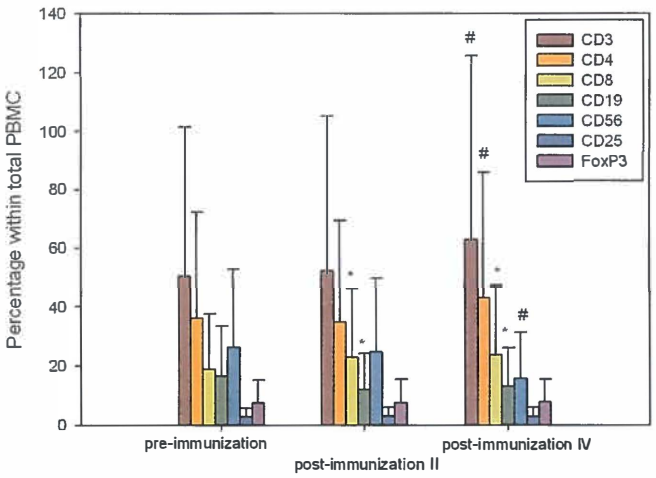
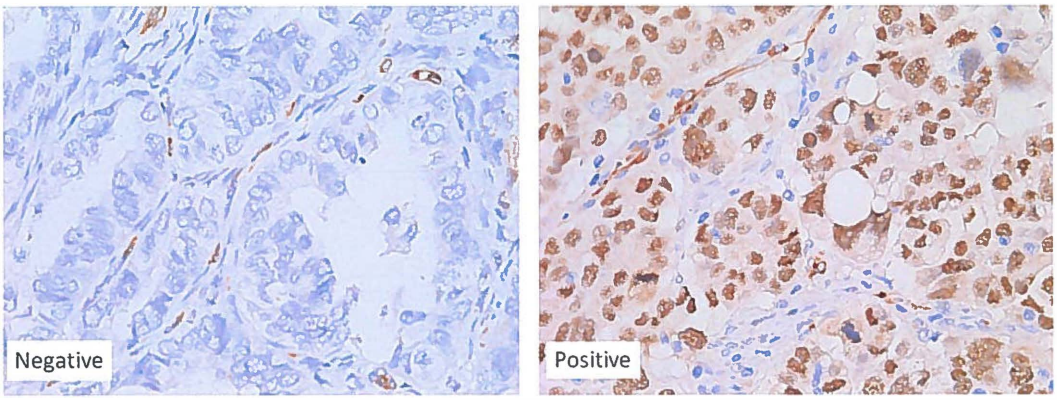
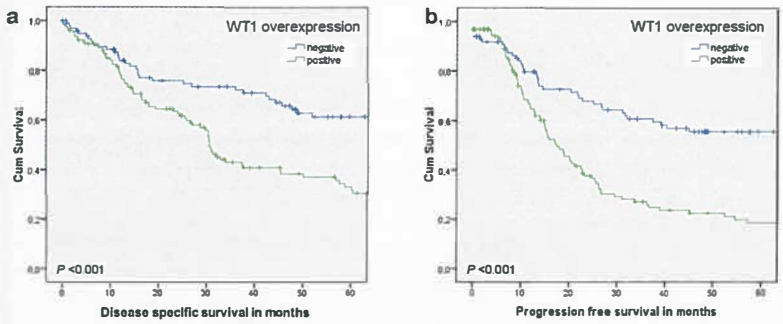


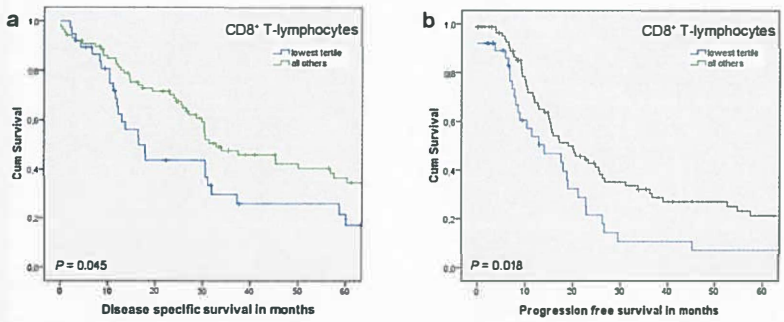
Chapter 3, ***Supplementary index*, Figure 2**



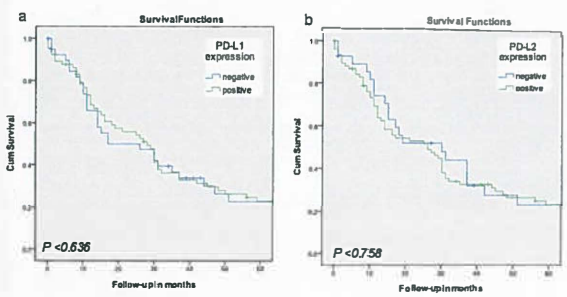
Chapter 6, **Figure 1**



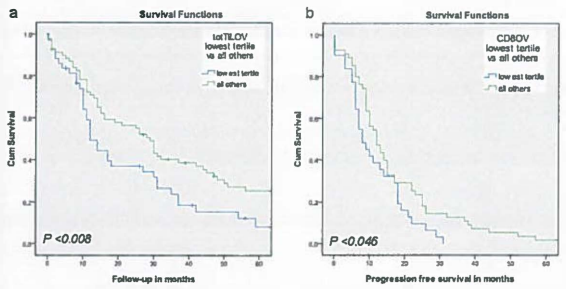
Chapter 6, **Figure 2**



Chapter 6, **Figure 3**



Chapter 7, **Figure 1**



Chapter 7, **Figure 2**

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CURRICULUM VITAE

Renee Vermeij werd op 1 juni 1983 geboren in Tilburg, als oudste dochter van Johan Vermeij en Vera van der Waarden. Zij groeide met haar twee zusjes op in Tilburg, Den-Bosch en Hengelo. Op het Twickel College in Hengelo haalde zij in 2001 haar atheneum diploma. Hierna begon zij met de studie geneeskunde aan de Rijksuniversiteit Groningen. In 2002 haalde zij haar propedeuse. In haar derde studiejaar begon zij met een onderzoeksproject bij de afdeling Voortplantingsgeneeskunde van het Universitair Medisch Centrum Groningen. Dit project resulteerde uiteindelijk in haar afstudeerscriptie. Renee heeft haar co-schappen in het Deventer Ziekenhuis gelopen. Het keuze co-schap Gynaecologie heeft ze in het Meander Medisch Centrum te Amersfoort gedaan. Na het behalen van haar artsexamen in de zomer van 2008 is Renee in het Universitair Medisch Centrum Groningen begonnen als artsonderzoeker bij de afdeling Gynaecologische Oncologie. Na haar promotie op 14 december 2011 zal zij in het Medisch Centrum Leeuwarden verder gaan met het eerste jaar van de opleiding tot gynaecoloog, welke zij in het voorjaar van 2011 gestart is.